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# Effects of ApoE(141 - 149)$^2$ Synthetic Tandem Repeat Peptide on Primary Cultures of Rat Sympathetic Neurons

by

**Valerie G. Templeman** ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

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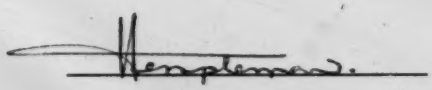

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ABSTRACT

Apolipoprotein E (apoE), a protein found in plasma and cerebral spinal fluid (CSF), has been implicated in Alzheimer's Disease. Previous studies by Crutcher and coworkers (1997) showed that an apoE(141 - 149)$^2$ dipeptide caused a decrease in cell viability in primary rat hippocampal neurons *in vitro*, but it was unknown at that time whether the actions of this dipeptide were on the axons alone or on the cell bodies. We utilized a compartmented culture system which allowed us to examine cell bodies and distal axons independently and found that apoE(141 - 149)$^2$ caused distal axon degradation by 24h but cell bodies/proximal axons were not affected. These data suggest that apoE peptides, or fragments, *may* exert deleterious effects on the axons but not necessarily on the cell bodies of brain neurons of patients suffering from Alzheimer's Disease.

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## Abbreviations

| | |
|---|---|
| Aβ | β-amyloid peptide |
| AD | Alzheimer's Disease |
| apo | apolipoprotein |
| apoA-1 | apolipoprotein A-1 |
| apoB | apolipoprotein B |
| apoC-1 | apolipoprotein C-1 |
| apoC-11 | apolipoprotein C-11 |
| apoC-111 | apolipoprotein C-111 |
| apoE | apolipoprotein E |
| apoER2 | apolipoprotein E receptor 2 |
| AraC | cytosine arabinoside |
| β-VLDL | β-very low density lipoprotein |
| BSA | bovine serum albumin |
| CC | centre compartment |
| CETP | cholesteryl ester transfer protein |
| CNS | central nervous system |
| CP | control peptide apoE(61 - 69)[2] |
| CSF | cerebral spinal fluid |
| Da | daltons |

| | |
|---|---|
| DMPC | dimyristoylphosphatidylcholine |
| DRG | dorsal root ganglion |
| DTT | dithiothreitol |
| *E.coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetate |
| HepG2 | hepatocytes G2 |
| $HDL_1$ | high density lipoprotein-1 |
| $HDL_C$ | high density lipoprotein -C |
| $HDL_P$ | high density lipophorin |
| HPLC | high pressure liquid chromatography |
| HSPGs | heparin sulfate proteoglycans |
| IDL | intermediate density lipoprotein |
| IL-2 | interleukin 2 |
| LDL | low density lipoprotein |
| LDLr | low density lipoprotein receptor |
| LHS | left hand side |
| LP | lipoprotein |
| LRP | low density lipoprotein receptor related protein |
| LTP | lipid transfer particle |
| mRNA | messenger ribonucleic acid |

| | |
|---|---|
| MTT | 3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide (from CellTiter 96™ Non-Radioactive Cell Proliferation Assay) |
| MW | molecular weight |
| NFT | neurofibrillary tangles |
| NGF | nerve growth factor |
| NMR | nuclear magnetic resonance |
| PBS | phosphate buffered saline |
| PNS | peripheral nervous system |
| RAP | receptor associated protein |
| RHS | right hand side |
| SDS | sodium dodecyl suflate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| rApoE | recombinant apolipoprotein E |
| TG | triglyceride |
| VLDL | very low density lipoprotein |

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*"Trust in the Lord with all your strength, and lean not on your own understanding, In all your ways acknowledge Him and He shall direct your path." Proverbs 3: 5,6*

# Chapter I. INTRODUCTION

## I.1 APOLIPOPROTEIN E AND ALZHEIMER'S DISEASE

By 1995 there were an estimated 3 - 4 million patients suffering from Alzheimer's Disease (AD) and it is believed that by the year 2000 almost 20 million people around the world will be struck with AD (Roses, 1995). Phenotypically AD is characterized by a gradual decrease in cognitive thinking, loss of memory, especially short term memory, and a decrease in attention (Higgins *et al.* 1997). These symptoms usually last between 3 - 20 years until complete functional incapacity occurs and death quickly follows (Roses, 1995). In 1993, Strittmatter and coworkers were able to show that apoE4, a protein found in senile plaques and nerurofibrillary tangles, is a risk factor for late-onset familial and sporadic AD (Rebeck *et al.* 1993; Strittmatter *et al.* 1993). Individuals with a homozygous genotype of apoE4 have a 4.5-fold greater risk of developing AD and show an earlier age of onset of the disease, at least 16 years earlier, on average (Strittmatter *et al.* 1993). Subjects carrying either one or two apoE2 alleles have a much lower risk of developing AD and the probability of developing AD increases somewhat later in life than their counterparts who carry apoE3 or apoE4 (Higgins, *et al.* 1997). It has been shown that individuals with even one apoE4 allele exhibit greater neurotoxicity and more highly developed senile plaques at autopsy than those with apoE3 (Nathan *et al.* 1994, Marques *et al.* 1996, Tolar, *et al.* 1997).

### I.1.1 CEREBRAL SPINAL FLUID LEVELS OF APOLIPOPROTEIN E AND ALZHEIMER'S DISEASE

The average concentration of apoE in the cerebral spinal fluid (CSF) of healthy control subjects is 5 - 10 μg/ml, which is approximately 10% of its plasma concentration (Hahne *et al.* 1997, DeMattos et al, 1998). Since apoE levels in the CSF are influenced by inflammatory diseases, such as AD, it has been suggested that apoE may be upregulated during the early stages of the disease due to inflammation and neuronal degeneration (Hahne *et al.* 1997). This in turn contributes to an increase in apoE concentration observed in some AD patients compared to age-matched control subjects (Hahne *et al.* 1997). However, this is a point of controversy as other studies indicate that rather than being increased, apoE is actually decreased 2-fold over normal controls (Aizawa *et al.* 1997). Previous studies have shown that CSF levels of apoE are significantly reduced in patients suffering from AD (Hahne *et al.* 1997). This decrease may be a reflection of co-aggregates with beta-amyloid (Aβ) peptide, as well as the reutilization of apoE/lipid complexes observed in neuronal regeneration and repair (Hahne *et al.* 1997).

It has been suggested (Hahne *et al.* 1997), that the increased levels of apoE seen in inflammatory diseases, such as AD, may be a useful diagnostic measure of AD. Because of the discrepancy between increased levels due to inflammation and the decreased levels due to co-aggregation with Aβ peptide and/or lipids during neuronal regeneration, levels of apoE do not appear to be a helpful diagnostic marker for AD (Hahne *et al.* 1997).

Notably apoE4, implicated in late onset familial and sporadic AD, binds with a higher affinity to Aβ peptide than either apoE2 or apoE3 (Castao *et al.* 1995, Rebeck *et al.* 1993). It is conceivable that once apoE is bound to Aβ peptide, cleavage by proteolysis occurs at the C-terminus of apoE (Castao *et al.* 1995) leaving the last amino acids bound to the Aβ peptide. The liberated N-terminus is not endocytosed by neuronal cells as only lipid-bound apoE is able to bind to its receptor. N-terminal apoE may interact by some unknown mechanism with neuronal cells leading to axonal degeneration. Increases in axonal debris, such as lipid and cholesterol, may ultimately lead to the production of more apoE.

### I.1.2 APOLIPOPROTEIN E AND TAU PROTEIN

Tau protein is a microtubule associated protein that gives stability to the microtubules in the axons of all neurons (Benzing and Mufson, 1995). ApoE3 has been shown to associate with tau protein and help to prevent tau hyperphosphorylation while apoE4 does not associate with tau and hyperphosphorylation occurs which leads to the formation of highly twisted neurofibrillary tangles (NFTs) (Strittmatter *et al.* 1994). Notably, apoE has been found in both neurons displaying NFTs and those that do not (Higgins *et al.* 1997). In some cases ApoE-like immunoreactivity has been shown to appear in areas of the AD brain displaying the most severe pathology (Higgins *et al.* 1997).

### I.1.3 APOLIPOPROTEIN E PROTEIN IN BOTH THE PLASMA AND CEREBRAL SPINAL FLUID

Human apolipoprotein E (apoE) is a 34 kDa protein that plays two important physiological roles: 1) it is able to impart structural integrity when bound to lipoprotein (LP) particles, and 2) once bound to a LP, it can serve as a ligand for specific LP receptors. Four such LP particles that associate with apoE are: 1) intermediate density lipoproteins (IDL) 2) chylomicrons, 3) chylomicron remnants, and 4) very low density lipoproteins (VLDL) (Weisgraber, 1994). ApoE plays a significant role in plasma lipid and cholesterol transport. It can be found in the CSF, along with ApoA-1 (Innerarity and Mahley, 1978), and is thought to function in the CSF in much the same way as it does in the plasma (Poirier, 1994).

ApoE consists of three isoforms, E2, E3, and E4, arising from three alleles: ε2, ε3, and ε4 of the APOE gene. The gene is 3.6 kb and is located on the long arm of chromosome 19 and contains three introns and four exons (Weisgraber, 1994). The differences among the three isoforms lies with two amino acids at positions 112 and 158. The E2 isoform has a cysteine residue at both 112 and 158; the E3 has a cysteine residue at 112 and an arginine residue at 158; and the E4 isoform has an arginine residue at both 112 and 158 (Weisgraber, 1994).

The three isoforms of apoE gives rise to six different genetic phenotypes: E2/E2, E3/E3, E4/E4, E2/E3, E2/E4, and E3/E4. ApoE3 is the most common

isoform worldwide at ~79% of individuals (Weisgraber, 1994), and is referred to as the parent form (Innerarity *et al.* 1983). ApoE2 and apoE4 account for 7% and 14% of the population, respectively (Weisgraber, 1994) ApoE isoforms have been implicated in various disease states. The apoE2 isoform has been implicated in the lipid disorder type III hyperlipoproteinemia (Innerarity *et al.* 1983) and as stated earlier, a positive correlation between the E4 isoform of apoE and the neurodegenerative disorder Alzheimer's disease was discovered by Strittmatter and coworkers in 1993.

## I.2. STRUCTURE AND FUNCTION OF APOLIPOPROTEIN E

ApoE is composed of 299 amino acids and was initially characterized in 1973 by Shore and Shore. They referred to apoE as the arginine-rich protein since it contains 34 arginines which is 11 % of the a.a. (Weisgraber, 1994). It can be separated into two independently folded segments: a 22 kDa domain containing the amino-terminus and a 10 kDa domain containing the carboxy-terminus. Both domains are functionally important (Weisgraber, 1994, Dyer *et al.* 1991) and are separated by a random chain, reported to be between either residues 165 and 200 (Rall *et al.* 1982) or 164 - 202 (Sparrow *et al.* 1985). This random chain is highly susceptible to proteolysis (Weisgraber, 1994).

## I.2.1 STRUCTURAL FEATURES OF APOLIPOPROTEIN E

The three dimensional structure of the N-terminal domain of apoE has been solved and consists of an antiparallel, four helix bundle (Wilson *et al.* 1991, Weisgraber, 1994) with an additional small 10 a.a. α-helix between helix one and two (Weisgraber, 1994). The extended helices range in length from 28 amino acids for helix 2, to 36 amino acids for helix 3 (Weisgraber, 1994). The four helix bundle consist of amphipathic helices, which direct their hydrophobic side chains toward the interior of the bundle (Weisgraber, 1994); further it also contains 28 leucine side chains. Most of the leucine residues occur at every seventh amino acid and have been shown to impart a leucine zipper-like structure (Weisgraber, 1994). This structure helps to stabilize the hydrophobic interface between helices 1 and 4 and helices 2 and 3 (Weisgraber, 1994). The N-terminal domain contains the receptor recognition site, and many of the amino acids along helix four (130 to 150) are rich in basic a.a. imparting an overall net positive charge on the helix bundle. It is believed that there is an ionic interaction between these positive charges and the negative charges on the various LP receptors (Sparrow *et al.* 1985, Weisgraber and Mahley, 1996).

The structure of the C-terminal domain is unknown, but has been predicted to contain three alpha helical segments (Weisgraber, 1994). This domain is highly hydrophobic and contains a region between residues 244 - 272 which is critical for interactions with the surface of LPs (Westerlund and

Weisgraber, 1993, Innerarity *et al.* 1983; Aizawa *et al.* 1997). The C-terminal domain has also been shown to be the region that binds to the amyloid beta (Aβ) peptide (Weisgraber and Mahley, 1996, Aizawa et al, 1997) found in senile plaques of Alzheimer's Disease ( Castao *et al.* 1995).

Full length apoE has been shown to self-associate into a tetramer when in a lipid-free aqueous state (Aggerbeck *et al.* 1988, Yokoyama *et al.*1985). While self association of other apolipoproteins is a function of concentration, (Weisgraber, 1994) apoE remains a tetramer over a broad range of concentrations (50 μg/ml to 15 mg/ml) (Yokoyama *et al.* 1985). Interestingly, tetramerization is not a prerequisite for lipid binding nor is this self-association apparent when the prerequisite four apoE proteins are bound to a lipid surface (Yokoyama *et al.* 1990, Weisgraber, 1994).

When apoE is treated with thrombin, which acts on the random chain between the two domains of apoE, two specific fragments are produced (Weisgraber, 1994, Tolar *et al.* 1997). When Aggerbeck and coworkers (1988) studied both fragments of apoE they determined that it was the C-terminal fragment that conferred the capability of forming a tetramer.

Once the C-terminus is bound to the lipid particle, it has been proposed that the N-terminus of apoE undergoes a conformational change from it's four-helix bundle conformation to an open structure (Weisgraber, 1994). This open structure is then able to expose its hydrophobic side chains and bind to LPs (Weisgraber, 1994) without disrupting the α-helical structure (Innerarity *et al.* 1984). This cross-talk, or communication, whereby structural changes occur in

the N-terminus after the C-terminus has been bound to the lipid surface is referred to as domain interaction (Weisgraber and Mahley, 1996). This interaction is believed to be critical, since apoE must be bound to the LPs if it is to serve as a ligand for LP receptors; it is believed that lipid-free apoE will not to bind to its receptor (Innerarity *et al.* 1979, Innerarity *et al.* 1983, Weisgraber, 1994)

### I.2.2 RECEPTOR BINDING

ApoE plays a crucial role in LP metabolism in many tissues and this role is directly related to the recognition of apoE by various cell surface receptors. Three of the receptors that bind apoE with high affinity are: 1) low density LP receptor (LDLr) (Innerarity and Mahley, 1978), 2) low density LP receptor-related protein (LRP) (Herz and Willnow, 1994), and 3) the apoER2 receptor found in the brain (Kim *et al.*1996).

The LDL receptor, characterized in 1976 by Brown and Goldstein, contains seven imperfect repeats of 40 amino acids that are cysteine-rich (Dyer *et al.* 1995). These cysteines are believed to be the critical residues for ionic interactions between LDLr and apoE- containing LPs (Weisgraber, 1994, Dyer *et al.* 1995). Interestingly, both apoE3 and apoE4 have been shown to bind equally well to both LDLr and LRP, but apoE2 binds with a 100-fold lower affinity (Guillaume *et al.* 1996, Higgins *et al.* 1997). Four apoE molecules are able to bind to one LP thereby exposing their positively charged clusters, and

consequently binding to one receptor with its multiple negatively-charged regions ( Dyer *et al.* 1995).

LRP is a cell-surface protein that bears a strong resemblance to the LDLr and has been shown to bind to and cause endocytosis of apoE-enriched lipoproteins (Herz *et al.* 1988). Like the LDLr, LRP also contains multiple (31) cysteine-rich regions which bind apoE. However, unlike LDLr, it does not bind to LDL (Strickland *et al.* 1990, Dyer *et al.* 1991). LRP has been detected in numerous tissues throughout the body, such as the liver, where it is believed that hepatocytes utilize LRP to internalize apoE-containing chylomicron remnants (Weisgraber *et al.* 1983, Hussain *et al.* 1991). LRP has also been found in various cells including T-lymphocytes of the immune system (Dyer *et al.* 1991, Dyer *et al.* 1995) and nerve cells including: a) hippocampal pyramidal neurons (Rebeck *et al.* 1993), and b) granule cells of the dentate gyrus (Rebeck *et al.* 1993). Conversely, LDLr has been localized in the brain primarily to neutrophils and astrocytes (Tolar *et al.* 1997). Since brain neurons express LRP receptors (Higgins *et al.* 1997), it is conceivable that these receptors may be involved in receptor-mediated uptake of apoE-containing lipoproteins (Higgins *et al.* 1997). Of particular interest is the fact that neuronal LRP is never exposed to plasma lipoproteins with the exception of a breakdown in the blood-brain barrier, such as in the case of head trauma (Roses, 1997).

The receptor binding domain of apoE has been determined, through various immunological studies, using monoclonal antibodies or, by the use of

apoE fragments of varying lengths (Vogel *et al.* 1985). The following regions have been proposed as the receptor binding regions of apoE: residues 136 - 158 (Weisgraber, 1994), 140 - 150 (Dyer *et al.* 1995), 140 - 160 (Dyer *et al.* 1991), 136 - 150 (Weisgraber, 1994), and 136 - 160 (Weisgraber, 1994). It is generally now accepted that residues 140 - 150 are critical for receptor binding activity (Innerarity *et al.*1983, Weisgraber *et al.* 1983, Lalazar *et al.* 1988). Most importantly, this binding activity has been attributed to both the lysyl and arginyl residues in this region (Cardin *et al.* 1986). It appears that if lysyl or arginyl residues are subjected to chemical modification, the ability of apoE to bind to its receptor on cultured fibroblasts is abolished (Innerarity *et al.* 1983, Cardin *et al.* 1986). These studies have resulted in a model in which multiple interactions of lysine and arginine residues occur during receptor:protein interactions (Dyer *et al.* 1995).

The apoER2 receptor, discovered in 1996 by Kim and coworkers, resembles LDLr and LRP in that it contains five functional domains: 1) N-terminal ligand binding domain, 2) an epidermal growth factor domain, 3) an *O*-linked sugar domain, 4) a transmembrane domain, and 5) a cytosolic domain containing a signal for coated pit targeting (Kim *et al.* 1996). Human apoER2 also contains seven cysteine-rich regions that are highly similar to those of the LDL receptor (Kim *et al.* 1996). Surprisingly, human apoER2 appears to bind apoE-enriched β-VLDL *in vitro* with a higher affinity than either LDL or VLDL. ApoER2 is highly expressed not only in human placenta but also in human brain (Kim *et al.* 1996). The abundance of this newly discovered

apoprotein in the brain suggests that it may be involved in the uptake of apoE-enriched PLs such as the HDL-like particles found in CSF (Kim *et al.* 1996).

## I.3 LIPOPROTEIN ASSOCIATION

Apolipoprotein E has been shown to associate with a variety of LP particles namely VLDL, IDL, chylomicrons, and chylomicron remnants. Once apoE is bound to a LP particle it exerts two very specific functions: a) confers stability to these particles (Weisgraber, 1994), and b) is a ligand for receptor mediated endocytosis (Brown and Goldstein, 1978). Therefore, apoE is an essential element of LP metabolism.

### I.3.1 VERY LOW DENSITY LIPOPROTEINS AND APOLIPOPROTEIN E

Apolipoprotein E constitutes approximately 15% of total proteins found on cholesterol enriched VLDL [other proteins on VLDL are apoB, apoC-1, apoC-11, and apoC-111] (Roth *et al.* 1977). The apoE2 isoform, in association with VLDL, has been shown to play a role in familial type III hyperlipoproteinemia or familial dysbetalipoproteinemia (Havel and Kane, 1973). It is possible that apoE2 may not be able to function as a ligand for the following three apoE receptors: a) VLDL receptor, b) LDL receptor, and c) LRP, since patients that are homozygous for apoE2 show increased levels of

plasma cholesterol and have a tendency to suffer from atherosclerosis (Higgins *et al.* 1997).

### I.3.2 HIGH DENSITY LIPOPROTEIN$_1$

Mahley and associates (1974) characterized a high density lipoprotein subfraction in cholesterol/fat-fed animal models which contain apoE. They termed this subfraction "HDL-with apoE" which was later changed to $HDL_1$ for cholesterol-rich HDL (Boyles *et al.* 1985, Innerarity *et al.* 1979, Mahley, 1985). Interestingly, in humans, $HDL_1$ is much less prominent accounting for approximately 20% of total plasma cholesterol (Weisgraber and Mahley, 1980). A possible explanation for the low levels of $HDL_1$ in humans lies in the activity of cholesteryl ester transfer protein (CETP). CETP exchanges triglycerides for cholesteryl esters from HDLs to LDLs, IDLs or VLDLs (Weisgraber, 1994). In species, such as dogs, rats, or mice which do not possess CETP, the majority of their plasma cholesterol is found in the $HDL_1$ subclass (Weisgraber and Mahley, 1980). For those species such as humans and rabbits that possess higher levels of CETP, the need for $HDL_1$ is not as great since most of the cholesterol is transported in LDLs and therefore the levels of HDL-with apoE are low (Weisgraber and Mahley, 1980). Another subclass of HDL that contains only the apoE protein is $HDL_c$ (Mahley *et al.* 1977). $HDL_c$ is found in the plasma of cholesterol/fat-fed dogs and has been useful in the study of the

interactions between apoE and the LDLr *in vitro* (Mahley *et al.* 1977, Weisgraber, 1994).

### I.3.3 INTERMEDIATE DENSITY LIPOPROTEIN AND APOLIPOPROTEIN E

Once VLDL loses much of its triglyceride the apoE protein still remains bound. Since apoE is still in association with this depleted VLDL particle, it continues to impart structural integrity, and VLDL is then considered an intermediate density lipoprotein (IDL). ApoE serves as a ligand and binds to the LDLr located on the liver ( Weisgraber, 1990).

### I.3.4 CHYLOMICRONS AND CHYLOMICRON REMNANTS

Plasma apoE becomes associated with chylomicrons after the chylomicrons have been released from the intestine and begin to circulate. ApoE imparts structural stability to these extremely large LPs (Weisgraber, 1994). As chylomicrons release their dietary triglycerides and cholesterol to adipose and other tissues they become chylomicron remnants and lose some of their apoE. The remaining apoE bound to the chylomicron remnants serve as ligands for LRP situated on the liver. Interaction between apoE and LRP facilitate receptor-mediated endocytosis of the chylomicron remnant with resultant release of both triglycerides and cholesterol into the liver with eventual recirculation into the plasma of the lipids by way of VLDL ( Weisgraber, 1994).

## I.3.5 APOLIPOPROTEIN E AND DIMYRISTOYLPHOSPHATIDYLCHOLINE

ApoE, like all soluble apolipoproteins, is able to bind to phospholipid and form discoidal-shaped particles (Weisgraber, 1994). When apoE is combined with dimyristoylphosphatidylcholine, (DMPC) these structures are organized into a disc-shaped bilayer, with four apoE molecules oriented on the perimeter of the disc (Innerarity *et al.* 1979, Pitas *et al.* 1980). DMPC vesicles alone are not stable after 2 - 3 days and aggregate, even in dilute solutions (Roth *et al.* 1977). Unlike DMPC vesicles, the apoE/DMPC complexes do not aggregate or dissociate into free apoprotein and lipid and remain stable up to 6 days. This suggests, that apoE may stabilize the DMPC discs (Roth *et al.* 1977). Most importantly, when apoE is bound to DMPC it maintains its receptor binding abilities as Fisher and coworkers demonstrated, utilizing a competition assay with LDL particles and N-terminal apoE/DMPC (Fisher *et al.* 1997). Moreover, when apoE is bound to DMPC the LDLr binding activity of apoE is 10 - 100-fold greater than binding activities of LDLs (Innerarity *et al.* 1979).

ApoE consists of two unique segments: 1) a 22 kDa N-terminal fragment that contains the receptor binding domain and a heparin binding domain, and 2) a 10 kDa C-terminal fragment that contains the lipid binding region and a heparin binding region. Fisher and coworkers (1997) demonstrated that the N-terminal 1 - 183 amino acids of apoE retains its four helix bundle conformation in solution. Recombinant N-terminal apoE was able to successfully prevent aggregation of LDL particles when incubated with high

density lipophorin (HDLp) and lipid transfer particles (LTP), thereby establishing that this portion of apoE has the ability to bind to a lipid particle (Fisher *et al.* 1997). Finally, N-terminal apoE(1 - 183) was complexed with DMPC to form discs which successfully competed for binding sites on human skin fibroblasts with $^{125}$I-LDL particles (Fisher *et al.* 1997).

## I.4 APOLIPOPROTEIN E BIOCHEMISTRY

Plasma apoE is synthesized in a variety of tissues such as the liver, intestine, kidney, lung, spleen, ovary, brain, and testes (Dyer *et al.* 1991, Higgins *et al.* 1997), but by far, liver shows the highest levels (54 μg/g of liver tissue). Although it is produced in the brain, at much lower levels than in the liver (21 μg/g of brain tissue), the brain is still the second highest producer of apoE overall (Boyles *et al.* 1985). When comparing mRNA levels in the liver and brain, Elshourbagy and coworkers (1984), found a ratio of liver/brain of 3:1. Plasma levels of apoE protein are approximately 5 mg/dl (Weisgraber and Mahley, 1996). It is interesting to note that apoE is produced in tissues that are not considered to be involved in cholesterol homeostasis such as the kidneys, spleen, and brain (Dyer *et al.* 1991). In some of these tissues apoE has been shown to be involved in immunoregulation (Weisgraber, 1994), modulation of cell growth (Avila *et al.* 1982), and differentiation (Weisgraber and Mahley, 1996).

## I.4.1 IMMUNOREGULATION BY APOLIPOPROTEIN E

As stated above, apoE has been shown to be physiologically important in immunoregulation (Weisgraber, 1994). Avila and coworkers (1982) showed that both lipid-bound and lipid-free full-length apoE was able to inhibit the proliferation of mitogen-activated T lymphocytes. Dyer and associates showed not only T cell inhibition but also a decrease in the production of androgens by gonadotropin-stimulated ovarian and interstitial cells (Dyer *et al.* 1991, Crutcher *et al.* 1994). With respect to the mechanism of inhibition, there are a couple of possible explanations: a) apoE-containing lipoproteins have the ability to bind to LRP on lymphocytes and suppress the immunological response of these cells to mitogens (Boyles *et al.* 1985, Ignatius *et al.* 1986), and/or b) apoE may contain a specific domain(s) that interacts with the T lymphocytes mediating inhibition (Clay *et al.* 1995). Although the question of inhibition has not been answered yet, it is known that the inhibition of mitogen-stimulated lymphocytes by apoE is readily reversible (Dyer *et al.* 1991). ApoE appears to be involved in the regulation of the immune system and immunocytochemical studies have been used to identify apoE in specific cells of the peripheral nervous system (Boyles *et al.* 1985, Clay *et al.* 1995). If apoE is involved in the regulation of portions of the immune system, it may be possible that apoE is involved in regulating other cells throughout the body.

## I.5 RECOMBINANT N-TERMINAL (1 - 183) APOE3 AND APOE4

The N-terminal 183 amino acids of recombinant apoE (rApoE) incorporate not only the receptor binding domain, but also the first heparin binding site of apoE ( Vogel *et al.* 1985, Fisher *et al.* 1997). Fisher and coworkers (1997) completed a series of studies that tested the structural properties of this recombinant protein. Expression and isolation of rApoE from transformed *E. coli* resulted in a preparation that was ≥ 95% pure when analyzed on a reversed-phase HPLC (Fisher *et al.* 1997). Recombinant apoE3(1 - 183) had a molecular mass of 21,191 Da and corresponded to the estimated molecular mass of 21,193 Da (Fisher *et al.* 1997). Circular dichroism spectroscopy was performed and the measurement of ellipticity confirmed a protein with high α-helical content (Fisher *et al.* 1997). Co-sonication of rApoE3(1 - 183) and DMPC produced stacked, disc-like structures when seen under an electron microscope (Fisher *et al.* 1997). The dimensions and aggregations seen with the discs were in agreement with previous studies done in which Innerarity and associates used full-length apoE and DMPC to form lipid bilayer discs (Innerarity, 1983, Fisher *et al.* 1997).

It has been reported that the C-terminal portion of apoE associates with and binds to the LP particle (Weisgraber, 1994). To assess the ability of rApoE3(1-183) to bind to the surface of a LP, a binding assay was performed (Fisher *et al.* 1997). After extensive lipid loading of human LDL, hydrophobic patches were exposed resulting in the formation of LDL aggregates with

concomitant increase in turbidity (Fisher *et al.* 1997). Recombinant apoE3(1-183) was able to bind to the expanded lipid-loaded LDL particles retaining particle integrity and inhibiting aggregation in a concentration-dependent manner (Fisher *et al.* 1997).

Recombinant apoE3(1-183) was also tested for its receptor binding activity. Residues 140 - 150 of apoE are reported to be responsible for receptor binding but only if the apoprotein is associated with lipid ( Innerarity *et al.* 1983). Fisher and coworkers complexed rApoE3(1-183) with DMPC and studied its ability to compete with $^{125}$I-LDL for LDL binding sites on human skin fibroblasts. As expected, rApoE3(1-183) was able to effectively compete with $^{125}$I-LDL in a concentration-dependent manner (Fisher *et al.* 1997). Finally, NMR studies were done on the rApoE3(1-183) protein to confirm that lipid-free rApoE3(1-183) retains its global fold under the conditions studied (Fisher *et al.* 1997).

## I.6 SYNTHETIC APOE-DERIVED PEPTIDES

In 1991 Dyer and Curtiss undertook a series of studies in which they used a number of different apoE peptides to test the immunosuppressive effects of apoE *in vitro*. ApoE has been shown to inhibit mitogen- and antigen-stimulated lymphocyte proliferation (Dyer *et al.* 1991). This role of apoE is completely independent of its regulatory role as a ligand for the uptake of cholesterol-rich lipoproteins and appears to be regulated by monocyte-derived

macrophages or ovarian granulosa cells (Dyer *et al.* 1991). As lymphocytes do not express the LDL receptor, it is possible that apoE may be interacting with LRP.

### I.6.1 SPECIFIC REGIONS OF APOE CONTAIN INHIBITORY ACTIVITY

By trying to understand the mechanism of action of apoE on these two cell types and to identify the receptor involved in this interaction, Dyer and Curtiss (1991) utilized specific regions of apoE to determine the segments conferring the immunosuppressive activity. The following apoE peptides were evaluated: a) 93 - 112 monomer, b) 161 - 171 monomer, c) 172 - 182 monomer, d) 174 - 193 monomer, e) 141 - 155 monomer, f) 141 - 150 tandem repeat dimer, and g) 141 - 155 dimer (branched dimer and linear tandem repeat) (Dyer *et al.* 1991). The two tandem repeat peptides represented a linear repeat of the receptor binding segment of apoE (Dyer *et al.* 1991). All the above peptides displayed amphipathic properties with lysine and arginine residues on the positively charged hydrophilic face, and leucine residues on the hydrophobic face (Dyer *et al.* 1991, 1995). None of the peptides studied showed any evidence of aggregation over various concentration and temperature ranges (Dyer *et al.* 1995). The monomeric apoE synthetic peptides were inactive in their ability to suppress T-cell proliferation (Dyer *et al.* 1991, 1995). However, the apoE(141 - 155)$^2$ tandem repeat dimer and the apoE(141 - 155) branched dimer had potent anti-proliferative effects on T

lymphocytes, (Dyer *et al.* 1991) implying that a tandem dimer is necessary for inhibition. This anti-proliferative activity is confined to the receptor binding segment of apoE (Dyer *et al.* 1991).

### I.6.2 FEATURES NECESSARY FOR ACTIVITY OF THE APOE PEPTIDES

In 1995 Dyer and coworkers focused on the structure of the apoE peptides. Why were some peptides able to exert inhibitory effects on cells in culture while others had no effect? The α-helical structure of apoE(141 - 155)$^2$ tandem repeat peptide was found to be absolutely necessary for receptor interaction, but was not the only essential feature required for inhibition (Dyer *et al.* 1995). Another requirement was the preservation of the pattern of positively charged residues along the polar face of the α-helix (Dyer *et al.* 1995). Finally, by deleting the first four or the last five amino acids on the apoE (141-155)$^2$ tandem peptide Dyer and coworkers (1995) were able to determine that the amino-terminal residues were critical for receptor binding activity while the carboxyl-terminal amino acids had no effect on receptor interactions.

Dyer and coworkers (1995) proposed that it was the spacing of the positively charged residues in the α-helix that allowed interactions with the negatively charged amino acids of the receptor. Interestingly, the apoE(141-150)$^2$ tandem peptide was more active than apoE(141-155)$^2$ (Dyer *et al.* 1995). Therefore, they concluded that the last three aspartic acid residues of the apoE(141 - 155)$^2$ tandem peptide may actually interfere with the

peptide:receptor interaction (Dyer *et al.* 1995). The apoE(141-150)$^2$ and apoE(141-155)$^2$ tandem repeat peptides exhibited some of the biological characteristics of the full-length apoE protein in terms of interaction with LDLr or LRP (Dyer *et al.* 1995). These studies clarify that the lipid-free form of the apoE (141-150)$^2$ tandem repeat was able to interact with its receptor even though it has been reported that full-length lipid-free apoE does not interact with its receptor (Weisgraber, 1994). It may be possible that intracellular events could occur in other cell types since cytostatic or cytotoxic events took place as a result of treatment with apoE peptides.

### I.6.3 ARE THE CYTOTOXIC AND CYTOSTATIC EFFECTS OF APOE SPECIES SPECIFIC?

In 1995 Clay and coworkers found that amino acids 130 - 169 had both cytostatic and cytotoxic effects on IL-2-dependent T lymphocytes. The monomeric peptides, encompassing amino acids 130 - 149 and 130 - 155, were able to inhibit cell proliferation without leading to cell death (Clay *et al.* 1995). By comparing the biological activity of the apoE peptides, using mouse or human IL2-dependent cell lines, it was determined that apoE(130-169) not only inhibited proliferation but also lead to significant cell death (Clay *et al.* 1995). Interestingly, an apoE peptide containing Cys → Arg substitution at residue 158, a normal consequence of apoE2 production, was also used to test the isoform specific activities of apoE2 and apoE3 peptides. It was

determined that the E2 isoform was able to inhibit IL2-dependent proliferation but demonstrated much lower levels of cytotoxic activity when compared to the apoE3(130-169) peptide (Clay *et al.* 1995). As a result Clay and coworkers concluded two very important points: 1) the effects seen with apoE peptides are not species specific, and 2) the results are not an artifact of the *in vitro* culture system.

### I.6.4 THE AMINO TERMINAL PORTION OF APOE(130-169) PEPTIDE PRODUCES THE CYTOTOXIC ACTIVITY

By examining both the amino and carboxyl portions of the apoE(130-169) peptide, it was determined that the amino terminal 130 - 149 residues induced approximately 20% of the cytotoxicity observed (Clay *et al.* 1995). The carboxyl 150 - 169 residues exerted no cytotoxic effects (Clay *et al.* 1995). This suggested that residues 141 - 149 of apoE were responsible for both the suppression of mitogen-activated naïve T cells and the cytotoxicity of both mouse and human cell lines (Clay *et al.* 1995).

### I.6.5 APOE(141 - 149)$^2$ IS APPROXIMATELY 20% MORE CYTOSTATIC AND CYTOTOXIC THAN APOE(141 - 155)$^2$

The branched dimeric apoE(141-155)$^2$, tandem repeat peptide, apoE(141 - 155)$^2$, and extended monomeric peptide apoE(130 - 169), were all able to exert potent cytotoxic activity when exposed to both mouse and human IL2-dependent cell lines (Clay *et al.* 1995). The overlapping region, consisting

of residues 141 - 149, situated between the functional peptides of apoE(130 - 169) and apoE(141 - 155)$^2$, was responsible for both cytostatic and cytotoxic activities (Clay *et al.* 1995). Based on this result, a tandem repeat of apoE(141-149)$^2$ was synthesized, which was found to be approximately 20% more cytostatic and cytotoxic than the apoE(141-155)$^2$ tandem repeat peptide (Clay *et al.* 1995).

### I.6.6 POSITIVE CHARGES ON THE APOE(141 - 149)$^2$ TANDEM REPEAT PEPTIDE ARE NECESSARY FOR ACTIVITY

Clay and associates (1995) tested the hypothesis that perhaps it was the positive charges on the residues between 141 - 149 that were responsible for both cytostasis and cytotoxicity activities. By using heparin, an anionic agent that interacts with apoE at the heparin binding sites: 142 - 147 and 211 - 243 (Cardin *et al.* 1986), they were able to demonstrate a decrease in the cytotoxic effects of apoE(141-155)$^2$ tandem repeat (Clay *et al.* 1995). The results demonstrated that the positive residues were involved in the cytotoxic activities seen in both mouse and human IL2-dependent cell lines (Clay *et al.* 1995). To confirm this observation, the second heparin binding domain of apoE, apoE(211 - 243), was synthesized. This peptide did not cause an inhibition of cell proliferation nor did it cause cell death (Clay *et al.* 1995).

### I.6.7 APOE PEPTIDES DO NOT HAVE AN EFFECT ON T LYMPHOCYTES THROUGH AN INTERACTION WITH THE CELLULAR MEMBRANE

The question of whether apoE peptides had an effect on T lymphocytes through cellular membrane interactions rather than with the apoE receptor was then addressed. Human fibroblasts, HepG2 cells, human uterine epithelial Ishikawa, or human and sheep erthroycytes were examined for cell lysis after exposure to apoE peptides (Clay *et al.* 1995). Cell lysis did not occur as a result of this interaction therefore the cytotoxic peptides were not hemolytic (Clay *et al.* 1995).

## I.7 STUDY OF APOE PEPTIDES IN BOTH PERIPHERIAL NERVOUS SYSTEM AND CENTRAL NERVOUS SYSTEM CELLS *IN VITRO*

ApoE is produced in the CNS by glia cells and has been found in both senile plaques and neurofibriallary tangles of Alzheimer's Disease (AD). Although the function of apoE in the CNS is still unclear, one hypothesis is that apoE may be a chaperone for the β-amyloid protein wherein apoE binds to Aβ peptide and ultimately this combination leads to an increase in amyloid plaques seen in AD (Sanan *et al.* 1994). Conversely, it has been proposed that apoE may not be a chaperone for β-amyloid protein (Strittmatter *et al.* 1993), but rather apoE4, full-length of E4 (Crutcher *et al.* 1994), both full-length and the 22 kDa N-terminus of apoE4, have been shown to cause neurite degeneration and cell death (Crutcher *et al.* 1994, Tolar *et al.* 1997). In either case, the pathological events leading up to AD are still unknown.

### I.7.1 EFFECTS OF APOE(141 - 149)$^2$ ON EXPLANTS OF CHICKEN SYMPATHETIC GANGLIA *IN VITRO*

Crutcher and coworkers tested the effects of apoE peptides on explants of embryonic chicken sympathetic ganglia *in vitro* (Crutcher *et al.* 1994). Extensive neurite degeneration was seen when apoE(141 - 155)$^2$ tandem repeat peptide, apoE3 (130 - 169), apoE4 (130 - 169), and apoE2(130 - 169) peptides were applied to chicken sympathetic explants (Crutcher *et al.* 1994). No neurite degeneration or decreased neurite extension was observed with purified full-length, delipidated apoE3 or the apoE(141 - 155) monomer peptides when compared to treatment with nerve growth factor only (Crutcher *et al.* 1994). Although the explants remained attached to the culture dish after the addition of 1 or 2 μM apoE(141 - 155)$^2$ tandem peptides, beading of neurites was observed after 15 - 20 min and by 20 h exposure, the neurites were completely destroyed (Crutcher *et al.* 1994). At 4 μM concentration, all the explants detached and floated (Crutcher *et al.* 1994). It was suggested that although a direct effect on cell bodies cannot be ruled out, the primary action of the apoE(141 - 155)$^2$ tandem peptide may be directly on the neurites (Crutcher *et al.* 1994).

It is noteworthy that although these tandem peptides exert toxic effects on neurites, the longer peptides, incorporating residues 130 - 169, were also toxic, albeit at a much higher concentration (Crutcher *et al.* 1994). It may be possible that since these longer peptides contain all the structural properties necessary for interaction with the receptors, they may exert toxic effects on the

axons in the same manner as apoE(141 - 155)$^2$. The delipidated apoE3 protein displayed no neurotoxic effects on the chicken sympathetic explants but it is possible that other isoforms, such as apoE4, or higher concentrations of apoE3 protein may cause similar degenerative effects seen with the apoE(141 - 155)$^2$ tandem peptide. Notably, apoE protein does not accumulate in the CNS unless a brain injury occurs and inflammation develops, therefore, under normal physiological conditions apoE protein is decreased in the CSF (Carlsson *et al.* 1991). If an accumulation of apoE protein is not seen, then the possibility of apoE fragment exposure to neurons would be limited to physiological levels of brain apoE only and injury to neurites would be minimal. Since Alzheimer's Disease is an inflammatory illness, it is conceivable that an increase in apoE production with resultant accumulation would be seen in the brain with comparable increases in apoE peptide formation.

### I.7.2 THE 22 KDA THROMBIN CLEAVAGE FRAGMENT OF APOE

To answer the question of whether fragments of apoE protein are able to exert neurodegenerative effects in the AD brain, Marques and coworkers (1996) studied a thrombin cleavage fragment of apoE found in CSF of normal and AD patients. This 22 kDa fragment, corresponding to the N-terminal end of apoE, is one of the most abundant apoE fragments found in both fresh CSF and homogenates of postmortem human brain tissue (Marques *et al.* 1996). The 22 kDa fragment was examined for it's neurodegenerative effects on

dissociated embryonic chick sympathetic neurons *in vitro* and was found to be considerably neurotoxic (Marques *et al.* 1996).

### I.7.2.1 THE 22 KDA THROMBIN CLEAVAGE FRAGMENT EXERTS NEUROTOXICITY *IN VITRO*

The apoE4 isoform exhibited potent neurotoxic effects at a lower concentration when compared to the apoE3 isoform (Marques *et al.* 1996). Thus, it is conceivable that the 22 kDa fragment, found in the CSF, is a biologically potent apoE fragment that may exhibit neurotoxic effects in the AD brain (Marques *et al.* 1996). Interestingly, these proteolytic peptides of apoE are able to cause axonal beading and degeneration of axons, *in vitro,* in the lipid free state (Tolar *et al.* 1997), a fact unknown until now since previous studies inferred that apoE must be bound to a lipid particle in order to exert its effect (Weisgraber, 1994). By studying the neurotoxic effects of both the apoE peptides and the 22 kDa fragment of apoE, Marques and coworkers suggested that apoE may not be a chaperone to the Aβ peptide as stated by Strittmatter and coworkers (1993), but rather may play a more direct neurotoxic role in the pathogenesis of AD (Marques *et al.* 1996, Crutcher *et al.* 1997, Tolar *et al.* 1997).

## I.8 ACTION OF APOE PEPTIDES

If apoE is the catalyst for the development of AD, what are these peptides doing to the axons to exert their destructive effects? To answer this question Tolar and coworkers (1997) examined the toxic activities of apoE(141 - 149)$^2$ tandem repeat peptide, apoE(141 - 155)$^2$ tandem repeat peptide, apoE(141 - 149) monomer peptide, apoE(130 - 169) monomer peptide, and apoE(263 - 286) monomer peptide on dissociated embryonic chicken sympathetic neurons and embryonic rat hippocampal neurons *in vitro*. Both chicken sympathetic neurons and rat hippocampal neurons were cultured for 24 h and then treated with various apoE peptides for 20 h and assessed for cell viability. Approximately seventy-five percent of the cells experienced axonal beading and cell death within 20 h when exposed to the three apoE peptides (Tolar *et al.* 1997). The monomeric peptides, apoE(141 - 149) and apoE(263 - 286), had no effect on either cell types (Tolar *et al.* 1997). The question of decreased cell viability has not been answered yet but will be addressed later.

### I.8.1 HEPARIN AND HEPARIN SULFATE INHIBIT THE NEUROTOXIC ACTIVITIES OF APOE PEPTIDES

Since all three toxic apoE peptides contain both the receptor binding domain and the N-terminal heparin binding site of apoE, and because previous studies determined that heparin was able to inhibit the toxic effects of apoE peptides (Clay *et al.* 1995), Tolar and coworkers (1997) tested the effects of

heparin binding and the toxicity of the apoE peptides. Chicken sympathetic neurons and dissociated chicken cortical neurons were preincubated with either heparin or heparin sulfate followed by the apoE toxic peptides. Both heparin and heparin sulfate completely inhibited the neurotoxic effects of the peptides in both cell cultures (Tolar *et al.* 1997). This lead to the hypothesis that initial binding to the heparin binding sites of apoE may be a necessary step before receptor interactions can occur (Tolar *et al.* 1997, Zhong-Sheng *et al.* 1997, 1998).

### I.8.2 AN INTERACTION BETWEEN APOE PEPTIDES AND HEPARIN SULFATE PROTEOGLYCANS IS A NECESSARY STEP THAT LEADS TO NEUROTOXIC EVENTS

To address the question of whether the interaction between apoE peptides and heparin sulfate proteoglycans (HSPGs) were a necessary step that lead to the neurotoxic activities demonstrated with these peptides, Zhong-Sheng and coworkers (1997,1998) demonstrated that interactions between apoE-enriched LPs and LDLr or LRP was a two-step process. The first step involves the binding of apoE-enriched LPs to HSPG which in turn leads to the transfer from HSPG to either LDLr or LRP (Zhong-Sheng *et al.* 1997, 1998). By pretreating the sympathetic neurons with heparinase I, an enzyme that degrades the glycosaminoglycan moiety of HSPGs thereby generating a defective receptor, significant protection of the sympathetic neuronal cells could be seen after treatment with the toxic apoE peptides (Tolar *et al.* 1997). These results suggest that HSPGs may play an important role in mediating

axonal beading and cell death elicited by the neurotoxic apoE peptides (Tolar *et al.* 1997).

### I.8.3 LRP AND HSPGs HELP TO FACILITATE THE TOXIC EFFECTS OF APOE(141 - 149)$^2$ ON NEURONAL CULTURES

The question of whether toxicity may also be the result of an interaction between the various toxic apoE peptides and their receptor lead to the investigation of LRP. Chicken sympathetic neurons and chicken cortical neurons preincubated with either the receptor associated protein (RAP) or anti-LRP completely protected the neurons from the toxic effects of both the apoE(141-149)$^2$ tandem repeat peptide and the 22 kDa fragment of apoE4 (Tolar *et al.* 1997). Since RAP binds to the active binding site of LRP, these results suggest that both the apoE peptides and the 22 kDa fragment of apoE exert their neurotoxic effects on these neurons through the same receptor complex (Tolar *et al.* 1997). Therefore, not only is LRP involved, but HSPGs may also facilitate the first step in the destructive course of action seen with the neurotoxic apoE peptides.

## I.9 THESIS OBJECTIVE

As stated earlier, Crutcher and coworkers (1997) determined that the cytotoxic activity seen with apoE(141 - 149)$^2$ in chicken sympathetic neurons and rat hippocampal neurons, that lead to decreased cell viability, may be confined to the axons themselves, but they were unable to test this hypothesis. Therefore, the focus of this work was to examine the cytotoxic effects of apoE(141 - 149)$^2$, N-terminal apoE3(1 - 183), and N-terminal apoE4(1 - 183) on two different segments of primary cultures of rat sympathetic neurons: 1) cell bodies and proximal axons, and 2) distal axons only. To achieve this goal, a compartmented culture system was used to determine the location of action of the toxic peptides.

The compartmented culture system has been described in detail (Campenot *et al.* 1991, Campenot, 1992). This system of studying different sections of a neuron is ideal since it contains three separate compartments and bulk fluid flow between compartments is restricted. Treatments can be added to one or both of the distal axon-containing compartments, to the centre cell body/proximal axon-containing compartment or to all three compartments and measurements taken of the effects on either the treated compartments of the non-treated compartments.

Another advantage of using the compartmented culture is the ability to axotomize the neurons. By spraying a sterile stream of ice cold distilled water into the side compartments, the distal axons are essentially removed from the

cell bodies and proximal axons. Neurite regeneration can be seen in the side compartments within 24h after axotomy. After 48h, various treatments can be added to either sides or centre compartments and neurite extension measured. The compartmented culture was used in many of the experiments in this work.

### I.9.1 THE VALIDITY OF USING APOE(141 - 149)$^2$ TANDEM REPEAT PEPTIDES TO STUDY THE ACTIONS OF APOE PROTEIN ON NEURONS *IN VITRO*

The importance of determining the effects of full-length apoE protein in damaged and regenerating neurons has been born out by a multitude of studies by various labs through out the world (Guillaume *et al.* 1996, Higgins *et al.* 1997). Conversely the effects seen with various lengths of toxic apoE fragments have generated even more studies and have stirred up much controversy (Crutcher *et al.* 1994, 1998, 1997, Clay *et al.* 1995, Wang *et al.* 1998)

ApoE production by macrophages is increased after a crush injury in the PNS and, in the CNS, apoE is produced by glia cells, predominantly astrocytes, and is increased when inflammation or injury occurs. It has been reported that specific synthetic peptides containing the receptor binding domain, or portions thereof, of apoE are able to exert damaging effects on mouse cell lines, mouse IL2-dependent cytotoxic T cell line, and human IL2-dependent primary T cells (Clay *et al.* 1995). One such peptide is the synthetic apoE(141 - 149)$^2$ tandem repeat peptide. The apoE(141 - 149)$^2$ cytotoxic peptide contains nine a.a., in

tandem, from the receptor binding domain of apoE protein and is strongly basic. It not only contains four lysine and six arginine residues, but dispersed throughout this peptide are eight leucine residues which confers a leucine zipper-like activity (Fisher *et al.* 1997). Because of the ten positively charged residues on the α-helical peptide, it is conceivable that they may interact with the negative charges on the LDL or LRP receptors. Although others have shown that full-length apoE must be associated with a lipid source in order for binding and cell-mediated endocytosis to occur (Innerarity and Mahley, 1978, Weisgraber, 1994), others have shown that fragments of apoE, not associated with lipid, have cytostatic and/or cytotoxic effects on diversified cells *in vitro* (Tolar *et al.* 1997, Crutcher *et al.* 1994, 1997, 1998).

Granted the use of rat sympathetic neurons, of the PNS, is not physiologically relevant, with respect to studies of AD, but the possibility of apoE fragments causing damage to a PNS neuron *may* impart some insight into what could happen in the CNS when neurons are exposed to apoE fragments that are found in the CSF (Castao *et al.* 1995). Indeed, when Marques and coworkers (1996) used the apoE(141 - 149)$^2$ tandem repeat peptide or the 22 kDa thrombin cleavage fragment on fetal rat hippocampal neurons *in vitro*, they found that these neurons showed beading of axons and cell death similar to that seen in embryonic day 9 chicken sympathetic neurons.

# Chapter II MATERIALS AND METHODS

## II.1 MATERIALS

Dimyristoylphosphatidylcholine (DMPC) was from Sigma Chemical Co. (St. Louis, Mo.). Recombinant human apoE3(1-183)/pET, recombinant human apoE4(1-183)/pET plasmids and transformed *E. coli* were a kind gift from Carl Fisher (University of Alberta). ApoE(141-149)$^2$ tandem repeat peptide was a kind gift from Dr. Keith A. Crutcher (University of Cincinnati). ApoE(61-69)$^2$ tandem repeat peptide was made at the Alberta Peptide Institute (University of Alberta). Isopropyl β-D-thiogalactopyranoside (IPTG) was from Rose Scientific (Edmonton, Alberta). Standard culture media Leibovitz-15 $CO_2$ (Life Technologies, Inc.) was supplemented as described previously (Campenot, 1992). Rat serum was obtained from University of Alberta, Laboratory Animal Services. Nerve Growth Factor, 2.5S, was obtained from Cedar Lane Chemicals, Hornby, Ontario or Alomone Labs, Israel. Polyacrylamide gel electrophoresis reagents and P-6DG gel was obtained from Bio-Rad (Hercules, CA). Protein A-Sepharose CL-4B beads and Heparin Sepharose CL-6B beads were obtained from Pharmacia-LKB Biotechnology, Inc (Quebec, Canada). Phospholipid B Kit was obtained from Wako Pure Chemicals Industries, Ltd (Richmond, VA). BCA Protein Assay Reagent Kit was obtained from Pierce (Rockford, IL). CellTiter 96 Non-Radioactive Cell Proliferation

Assay (MTT) Kit was from Promega (Madison, WI). All other reagents were purchased from BDH, Fisher, or Sigma.

### II.1.1. BUFFERS AND SOLUTIONS

All buffers and solutions were prepared using analytical grade reagents in distilled deionized water.

## II.2 METHODS

All methods utilizing primary cultures of rat sympathetic neurons and expression and purification of recombinant apoE were done using sterile techniques.

### II.2.1 PREPARATION OF PRIMARY CULTURES OF RAT SYMPATHETIC NEURONS

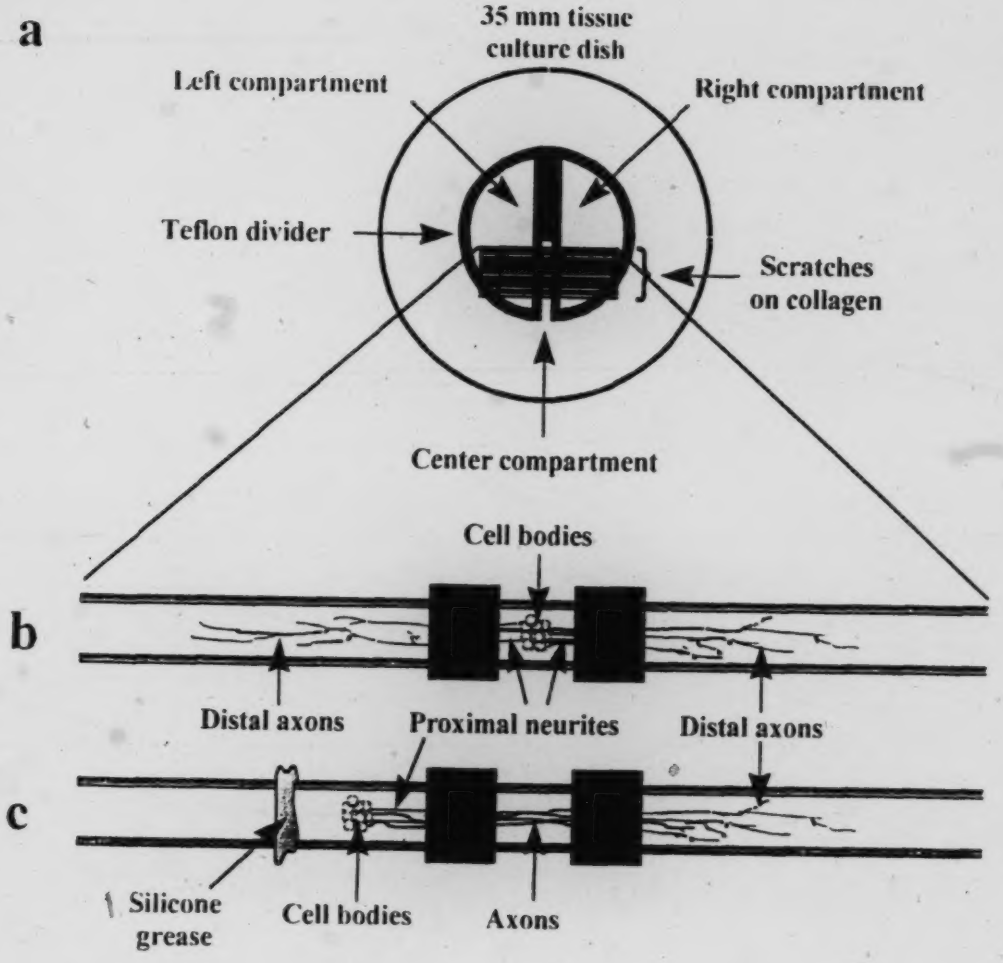
General procedures for preparing and culturing primary rat sympathetic neurons have been described previously (Campenot *et al.* 1991). Briefly, superior cervical ganglia were dissected from newborn Sprague-Dawley rat pups (University of Alberta farm). The ganglia were trypsinized ( 0.1% ) for 20 minutes, after spinning the ganglia and washing 2X with sterile PBS, plating medium was added to stop the enzymatic reaction and ganglia were mechanically ground in a special glass syringe equipped with a small window

on the side, to allow for insertion of ganglia, and a 200 mesh stainless steel screen (200/inch) at the bottom of the syringe which allows for more complete separation of the cells when the syringe plunger is applied to the screen. Following dissociation, cells were plated on rat tail collagen-coated 24-well plates at a concentration of 1-ganglia/well or in the centre compartment of the compartmented culture (Fig. 1) at a concentration of (.6 ganglia /dish). Each compartment contains a completely separate fluid environment with essentially no bulk fluid flow among any of the compartments. Within 2 days of growth the side compartments contained only the distal axons while the centre compartment contained only the cell bodies and proximal axons. Non-neuronal cells in the centre compartment were eliminated within the first 6 days by adding 10 μM cytosine arabinoside (AraC); an antimitotic which inhibits cell division. 2.5% rat serum, containing nutrients specifically required by rat sympathetic neurons, and 1 mg/ml ascorbic acid, a cofactor in the synthesis of noradrenaline, and part of the regular components found in the fluid bathing sympathetic neurons, were also added to the cell body-containing compartment. NGF [10 ng/ml] was added to the cell body/proximal axon-containing compartment since primary cultures of rat sympathetic neurons are dependent on NGF for survival (Levi-Montalcini and Booker, 1960). Both side compartments were supplied with 100 ng/ml NGF throughout the initial growth period to encourage axonal growth along the previously scored tracks, under the teflon divider and through the grease barrier into the side compartments.

Figure 1. Schematic representation of the Compartmented Culture System

a) Entire culture system, b) Single track showing neurons plated in the centre compartment, and c) Single track showing neurons plated in the left hand compartment with distal axons growing into both the centre and right hand compartments. Cell bodies and proximal axons grow in the centre compartment, which is approximately 1 mm wide, and distal neurites extend under the teflon divider, through the silicon grease barriers and into both the left or right side compartments. Twenty tracks, approximately 200 µm wide are formed on the floor of a 35-mm plastic tissue-culture dish coated with rat tail collagen. These 20 scratches represent areas of the dish in which the collagen substratum has been removed. Growing neurites will not cross over this dried area and consequently axons grow in a relatively straight orientation along the tracks.

*Diagram used with permission from E. Posse de Chaves (University of Alberta)*


a
35 mm tissue culture dish
Left compartment
Right compartment
Teflon divider
Scratches on collagen
Center compartment
b
Cell bodies
Distal axons
Proximal neurites
Distal axons
c
Silicone grease
Cell bodies
Axons

After 6 days the AraC and NGF were eliminated from the growth media in the centre compartment and only 2.5% rat serum and 1.0 mg/ml ascorbic acid were given to the cell body/proximal axon-containing compartment. All culture media was changed every 4 - 6 days. In most cases cells were allowed to grow for 14 days before the start of any experiment. Generally axons would increase in length by ~ 1mm/day.

Mass cultures of rat sympathetic neurons were given all the same necessary nutrients as with the compartmented cultures but rather than 10 ng/ml NGF, they were given 100 ng/ml. This level of NGF was necessary to promote neuronal growth and denser cultures in mass cultures.

### II.2.2 MTT ASSAY TO MEASURE MITOCHONDRIAL ACTIVITY

This proliferation assay was used to measure mitochondrial activity in both the cell body/proximal axon-containing compartment and the distal axon-containing compartments. MTT assay provides a useful measure for determining cell viability. This assay is based on the ability of active mitochondria to convert a tetrazolium salt, in the dye solution of the CellTiter 96™ Non-Radioactive Cell Proliferation Assay kit, into a formazan product. Finally by adding a solubilization solution to solubilize the culture material and eliminate the interference of phenol red in the culture media, a reading at 570 nm was recorded using an ELISA reader. Absorbance is directly proportional to the number of active mitochondria/ cells in the culture.

## II.2.3 BACTERIAL EXPRESSION AND PURIFICATION OF RECOMBINANT APOE3(1-183) AND APOE4(1-183)

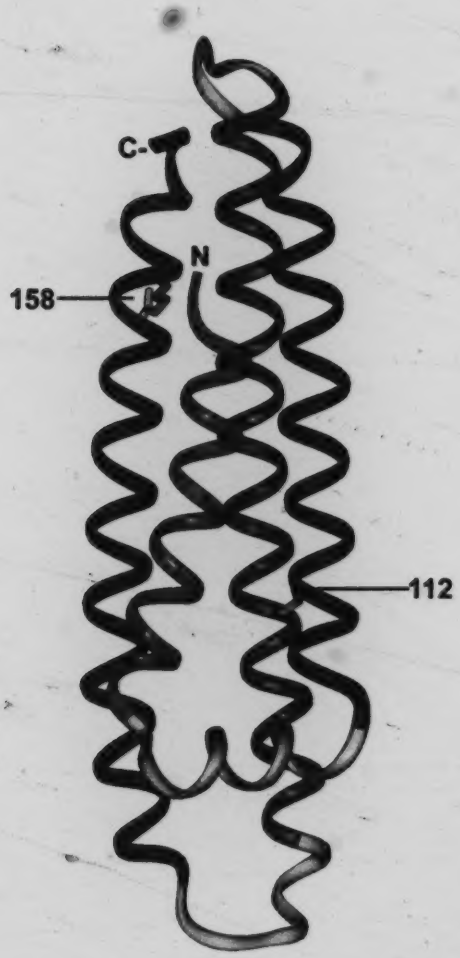
Saturated overnight cultures of *E. coli* transformed with either apoE3(1-183)/pET or apoE4(1-183)/pET vectors grown in 2x YT media ( bacto-tryptone, bacto-yeast extract, NaCl and 50 mg/ml ampillicin) were transferred into M9 minimal media containing 40% glucose, 1 M $MgSO_4$, and 1 M $CaCl_2$ at a 1:50 dilution and grown at 30 °C in an orbital incubator shaker, Gyromax 703, to an $OD_{600}$ of 0.6 - 0.7 (Fisher, *et al.*, 1997). IPTG [2 mM] was added to induce the production of the recombinant N-terminal apoE protein (Fig. 2). Approximately 4 - 5 hours later *E. coli* was pelleted by centrifugation at 10,000 x g, 4 °C for 10 minutes in a Beckman Model J2-21 Centrifuge and the supernatant stored at 4 °C overnight. The supernatant was concentrated using an ultrafiltration Watson-Marlow 313S concentrator and transferred to an Omegacell 150 ml stirred cell (Filtron) until approximately 30 mls of concentrated solution was remaining. This solution was dialyzed against 20 mM $NaPO_4$ pH 7.0, and 150 mM NaCl to remove excess salt overnight at 4 °C. The dialyzed solution was spun for 10 minutes at 10,000 x g at 4 °C in a Beckman Model J2-21 Centrifuge to remove contaminating residual *E. coli* and subjected to Heparin-Sepharose CL-6B (Pharmacia Biotech) affinity column chromatography. The column was first washed with 150 mM NaCl to equilibrate the column, then the protein-containing solution was added. The column and sample were washed with 150 mM NaCl and the protein was eluted (flow rate = 1 mm/min) with 1 M NaCl

Figure 2. N-Terminal ApoE3(1 - 183) Ribbon Diagram

N-terminal apoE, (adapted from Wilson, *et al.*, 1991), is characterized by an extended four-helix bundle arranged in an antiparallel conformation. Positions 112 and 158 distinguish the three isoforms of apoE such that apoE2 contains a cysteine at both 112 and 158, apoE3 contains a cysteine at 112 and an arginine at 158, and apoE4 contains an arginine at both 112 and 158.

*Used with permission from Carl A. Fisher (Lipid & Lipoprotein Group, University of Alberta)*

Ribbon Diagram of N-Terminal ApoE3(1 - 183)



and fractions collected. The eluted sample was applied to a desalting Bio-Gel P-6 DG (Bio-Rad) column (flow rate = 1 mm/min) to remove as much excess salt as possible and flash frozen at -70 °C for 2 hours. Finally the desalted sample was lyophilized in a Labconco lyophilizer for 3 days and stored at -20°C.

### II.2.4 PREPARATION AND ISOLATION OF APOE(1-183)/DMPC DISCS

Lyophilized apoE sample was resuspended in 1X PBS and protein concentration was determined using a BCA protein assay kit. A measure of apoE(1 - 183) to DMPC was calculated at 1:3 (w/w) and DMPC was added to a 13 x 100 mm glass tube. The DMPC was dissolved in approximately 250 µl chloroform and dried by a constant supply of nitrogen gas over lipid/chloroform for approximately 1 hour. The resuspended apoE(1 - 183) was added to the dried thin layer of DMPC and warmed to 45 °C for 5 min, vortexed for 1 minute to achieve an even slurry then bath sonicated for approximately 30 minutes until clear and finally vortexed again for ~ 30 seconds. The apoE(1 - 183)/DMPC mixture was incubated in a water bath/shaker for 18 h at 24 °C. The over-night mixture was applied to a Sepharose 4B column that had been pre-saturated with phosphatidylcholine (1 mg/ml) to help prevent the discs from sticking to the Sepharose beads and 1 ml fractions were collected. Fractions containing both protein and lipid were determined by performing a crude protein assay using a spectrophotometer at 280 nm and a WAKO phospholipid B kit

respectively (Fig. 3). Fractions containing both protein and lipid were combined and concentrated using a Centricon Plus-20 Centrifugal Filter Device. An electron micrograph was done to ensure disc formation (Fig. 4). The apoE(1 - 183)/DMPC discs were stored at -4 °C.

### II.2.5 MEASUREMENT OF NEURITE EXTENSION AFTER AXOTOMY

Distal axons were axotomized by mechanical removal from both left and right side compartments with a spray of ice cold sterile distilled water delivered through a 22 gauge needle and 5 ml syringe. The water was removed and this process was repeated twice more. This efficiently removes all visible traces of axons from the side compartments without disturbing the cell bodies or proximal axons in the centre compartment. Base media containing 10 ng/ml NGF was then added to both side compartments and neurites allowed to regenerate before addition of apoE3(1-183), apoE4(1-183), apoE(61 - 69)$^2$ (Fig. 5), or apoE(141 - 149)$^2$ (Fig. 5). Base medium only was added to those cultures treated with anti-NGF antibody. Neurite regeneration was measured using a Nikon MD105 Optical Encoder affixed to the left-right axis of a mechanical stage of an inverted phase contrast microscope. An eyepiece cross hair in the microscope was used to measure the farthest extending neurite from the Teflon divider.

Figure 3. Gel Filtration Chromatography of ApoE3(1 - 183)/DMPC Discs

ApoE3(1 - 183)/DMPC discs were prepared as described in Chapter II and applied to a Sepharose 4B column ($V_o$= 10 ml, $V_E$=10 ml, and $V_T$= 20 ml) and 1.0 ml fractions collected using 1X PBS buffer. Closed squares correspond to apoE protein (measured at Absorbance $OD_{280}$ nm) and closed diamonds correspond to DMPC (measured at Absorbance $OD_{495}$ using a basic enzymatic calorimetric assay produced commercially by Wako, Richmond VA). Fractions 10 - 20, containing both apoE3(1 - 183) and DMPC, were combined and concentrated as described in Chapter II. Shown is a profile that is representative of 5 individual preparations.

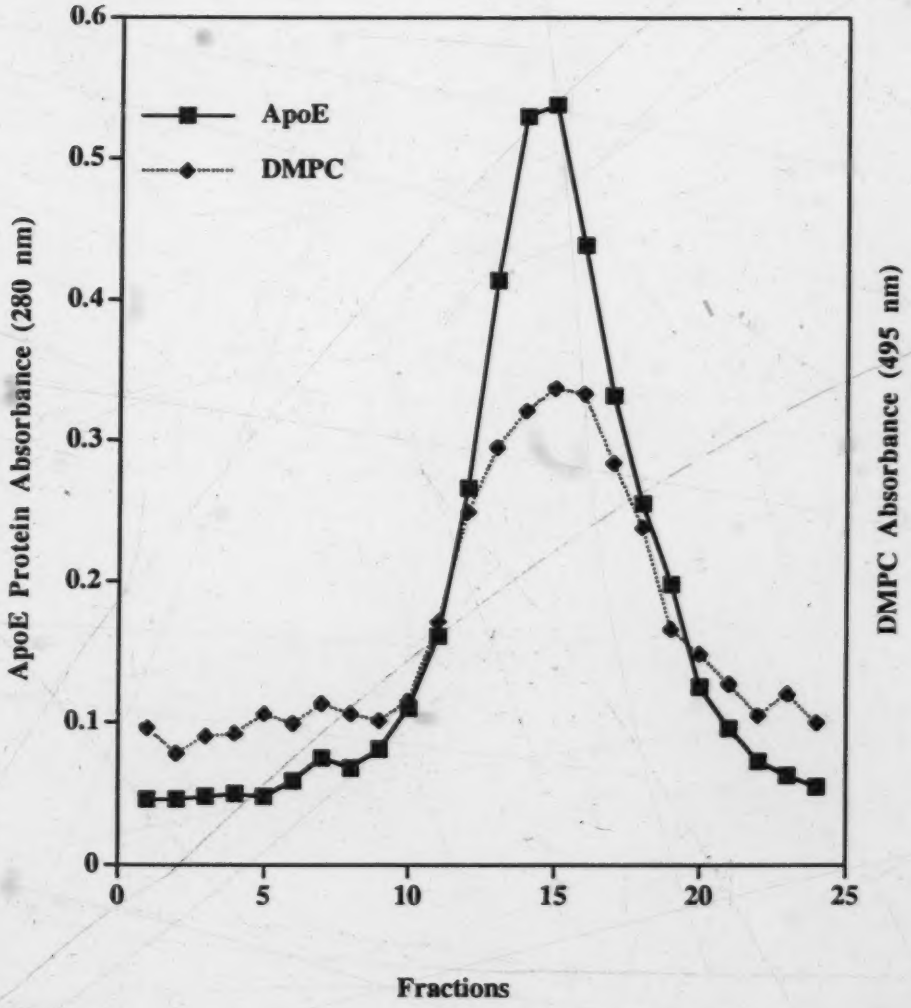

ApoE
DMPC
ApoE Protein Absorbance (280 nm)
DMPC Absorbance (495 nm)
Fractions
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25

Figure 4. Electron Micrograph of ApoE3(1 - 183)/DMPC Discs

Electron micrograph of apoE3(1 - 183)/DMPC disc complexes (See Fig. 3). Discs were stained with 2% NaPT stain and photographed using a Philips EM420 at 45,000X magnification. Enlargement = 135,000X magnification, 100 nm = 13.5 cm

**Electron Micrograph of apoE3(1 - 183)/DMPC discs**

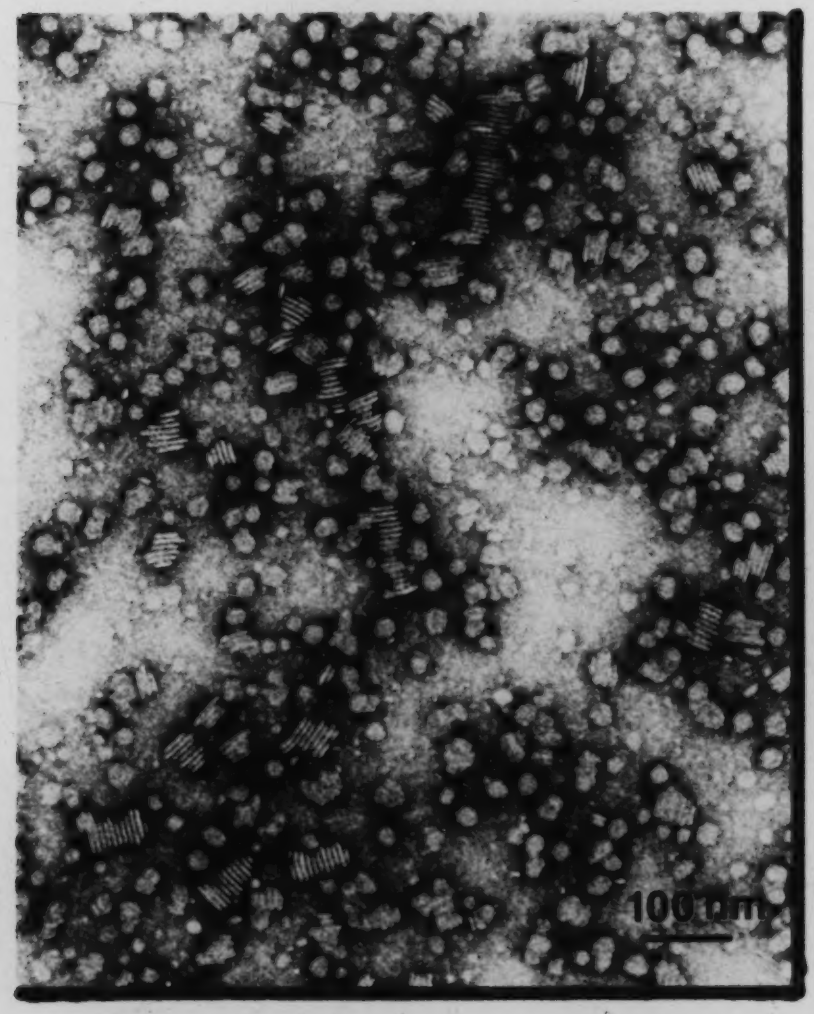


Figure 5. Linear Representation of ApoE(141 - 149)$^2$ and ApoE(61 - 69)$^2$

Synthetic tandem repeat peptides generated from two separate portions of the N-terminal ApoE protein. A) cytotoxic peptide apoE(141 - 149)$^2$ (kindly provided by Dr. K. Crutcher, U of Cincinnati) and, B) non-cytotoxic peptide apoE(61 - 69)$^2$ (The Alberta Peptide Institute, U of Alberta).

# NEUROTOXIC AND NON-NEUROTOXIC APOE PEPTIDES

**A) Neurotoxic apoE(141 - 149)[2]**

L R K L R K R L L L R K L R K R L L

**B) Non-Neurotoxic apoE(61 - 69)[2]**

R A L M D E T M K R A L M D E T M K

## III.1 RESULTS

### III.1.1 EXPRESSION AND PURIFICATION OF N-TERMINAL APOE3(1 - 183) AND N-TERMINAL APOE4(1 - 183)

The apoE vectors were expressed using four separate *E. coli* stocks. The isolated DNA was analyzed after restriction digest. As seen in Figure 6, inserts of 550 basepairs are observed with apoE3 (lanes 1,2) and 550 basepairs for apoE4 (lanes 4,5) as expected. Lane 3 shows the lambda DNA basepairs after HindIII digestion.

Using these vectors, N-terminal apoE3(1 - 183) and N-terminal apoE4(1 - 183) protein were expressed and purified as described in chapter II. This procedure results in relatively high purity as seen in Figure 7. Both purified forms of apoE(1 - 183) agree with the approximate molecular weight of 22 kDa. Finally, reverse phase analytical high pressure liquid chromatography (HPLC) was used to ensure purity (≥ 95%) of both truncated forms of apoE (Figs. 8 & 9).

### III.1.2 APOE3(1 - 183)/DMPC AND APOE4(1 - 183)/DMPC DISC FORMATION

Formation of DMPC discs with either apoE3(1 - 183) or apoE4(1 - 183) was accomplished according to Fisher and coworkers (1997) and as described in Methods, Chapter II. As stated previously, N-terminal apoE is able to bind to DMPC and form disc-shaped structures (Fisher *et al.* 1997). Four apoE particles bind to DMPC (Roth *et al.* 1977) and form stacked discs ranging

Figure 6. Plasmid Digest of ApoE3(1 - 183)/pET and ApoE4(1 - 183)/pET Vectors

Restriction digest, using MscI and HindIII restriction enzymes, of apoE3(1 - 183)/pET (lanes 1 & 2) and apoE4(1 - 183)/pET vector (lanes 4 & 5) (a kind gift from C. Fisher, U of Alberta). Lane 3 represents Lambda DNA marker digested with HindIII. Digested DNA was visualized by ethidum bromide staining followed by UV visualization. Inserts depicting both apoE3(1 - 183) and apoE4(1 - 183) can be seen at 550 basepairs as expected (See arrow)(Fisher *et al.* 1997). Shown is a photograph representative of one preparation.

**Agarose gel of N-Terminal apoE3(1 - 183) and N-Terminal apoE4(1 - 183) inserts**

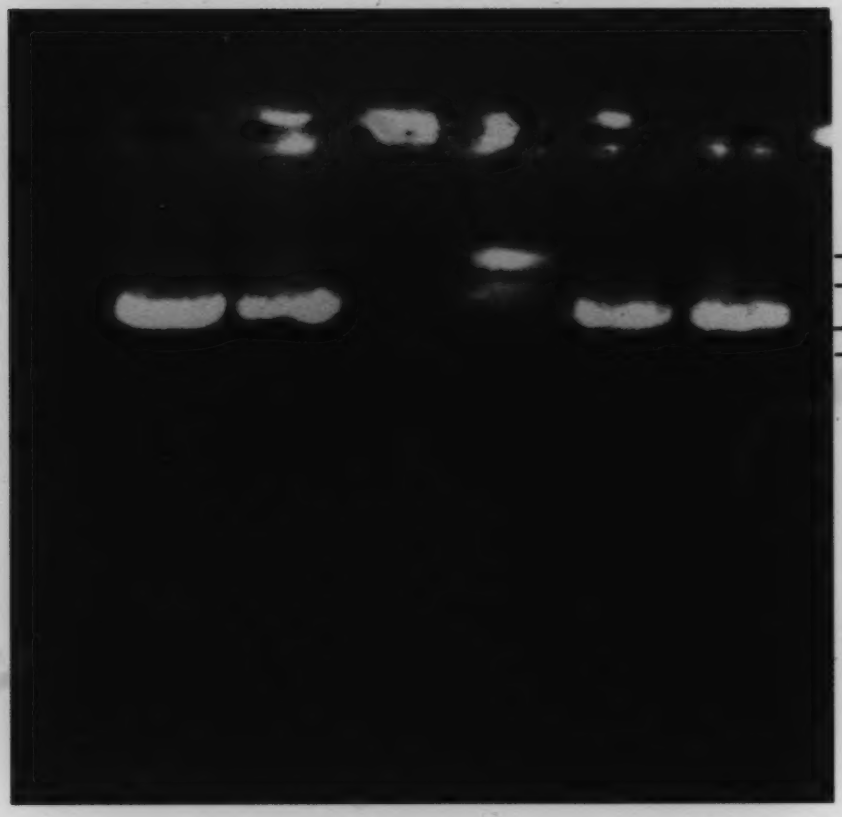


Figure 7. SDS-PAGE of Recombinant N-terminal ApoE3(1 - 183) and Recombinant N-terminal ApoE4(1 - 183)

Two μg of either apoE4(1 - 183) (lane 2) or apoE3(1 - 183) (lane 3) were prepared and separated on a 12% gel by SDS-PAGE as described in Methods. Lane 1 is the low weight molecular markers. ApoE4(1 - 183) (Lane 2) and apoE3(1 - 183) (Lane 3) can be seen at approximately 22 kDa. Shown is a gel representative of > 20 individual preparations.

**SDS-PAGE of Recombinant N-Terminal apoE3(1 - 183) and N-Terminal apoE4(1 - 183)**

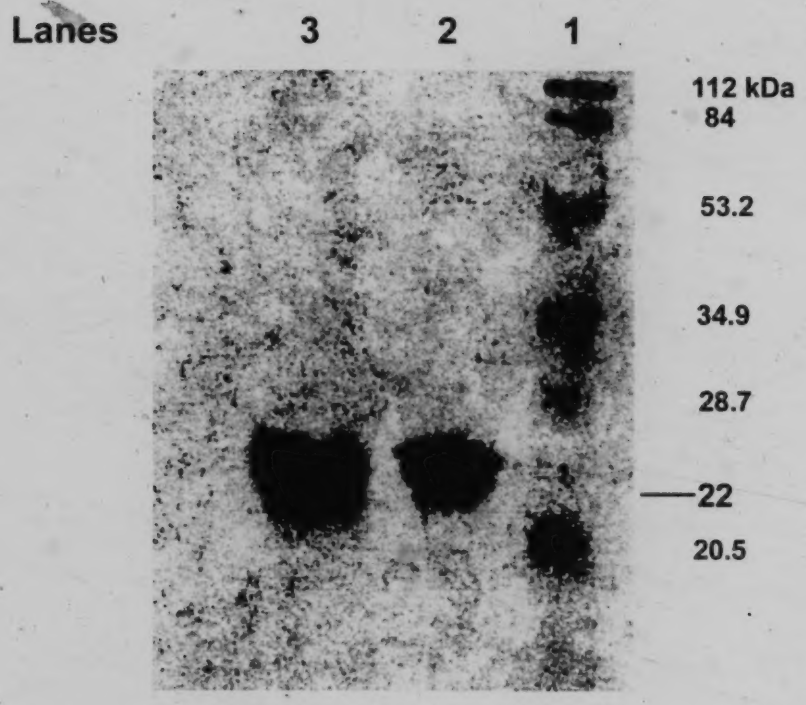


Figure 8. Reverse Phase Analytical Profile of Recombinant ApoE3(1 - 183) Using High Pressure Liquid Chromatography (HPLC)

N-terminal recombinant apoE3(1 - 183) was expressed as described in Methods. Rate of 2 %/minute of solution B (acetonitrile with 0.05% TFA) shows a major peak at approximately 30 minutes corresponding to the elution of rApoE3(1 - 183). Diagram is representative of 3 different preparations.

Analytical Profile of N-Terminal ApoE3(1 - 183)

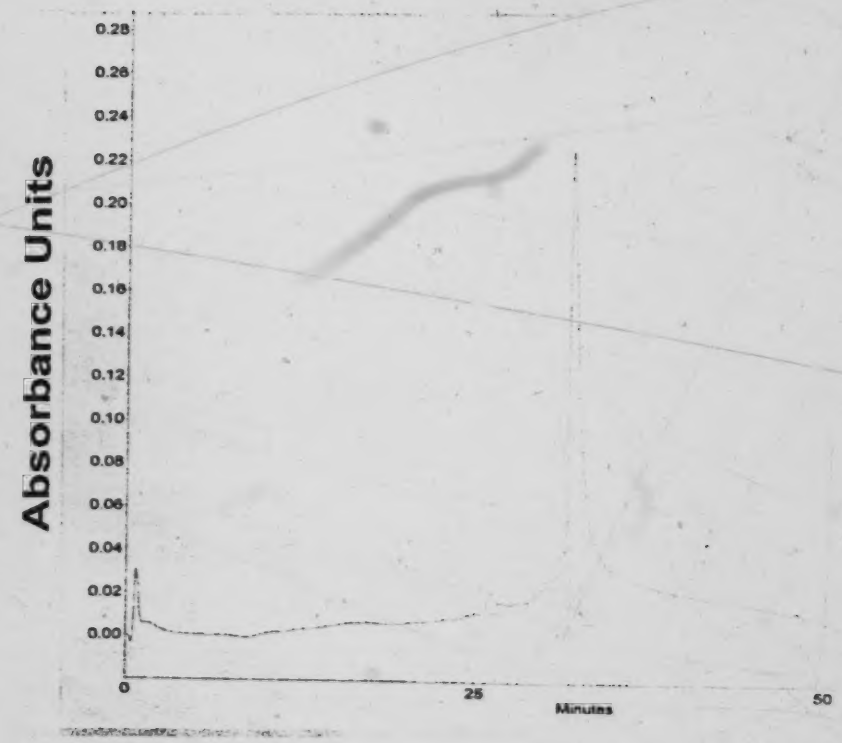
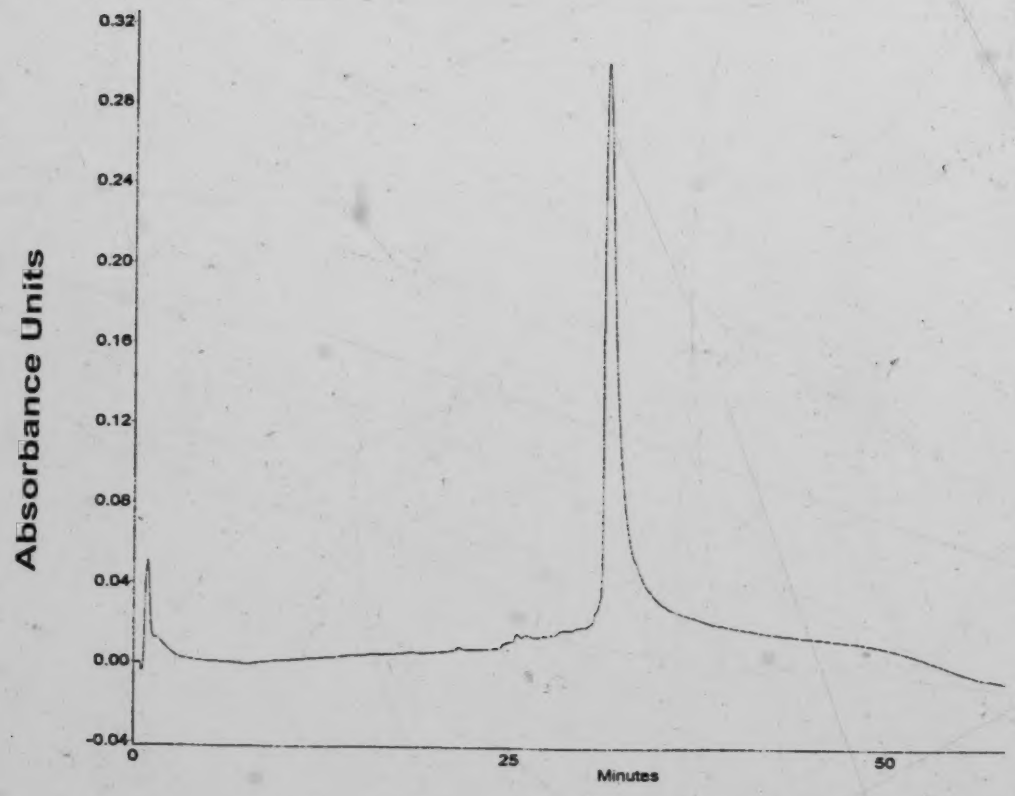


Figure 9. Reverse Phase Analytical Profile of Recombinant apoE4(1 - 183) Using High Pressure Liquid Chromatography

N-terminal recombinant apoE4(1 - 183) was expressed as described in Methods. Rate of 2%/minute of solution B (acetonitrile with 0.05% TFA) shows a major peak at approximately 30 minutes corresponding to elution of recombinant apoE4(1 - 183). Diagram is representative of 3 different preparations.

**Analytical Profile of N-Terminal ApoE4(1 - 183)**



from several to more than 26 discs (See Fig. 4). An elution profile from a Sepharose 4B column of apoE3(1 - 183)/DMPC discs can be seen in Figure 3.

## III.2 COMPARTMENTED CULTURES OF PRIMARY RAT SYMPATHETIC NEURONS, AXOTOMY AND MEASUREMENTS OF NEURITE REGENERATION

It is known from previous studies that apoE(141 - 149)$^2$ tandem repeat peptides cause a decrease in cell viability when applied to mass cultures of primary chicken sympathetic neurons or rat hippocampal neurons (Crutcher *et al.* 1994, Tolar *et al.* 1997). What is not known, however, is whether the actions of this synthetic peptide are on the distal axons only, cell bodies only, or on both axons and cell bodies together. By utilizing the compartmented culture it may be possible to localize the actions of apoE(141 - 149)$^2$ to either the distal axon-containing compartments or the cell body/proximal axon-containing compartment.

Trypsinized and mechanically dissociated superior cervical ganglia were plated in the centre compartment of the compartmented culture. Clusters of cell bodies and proximal axons could be seen in the centre compartment after one day in culture while distal axons were visible in the side compartments two days after plating. As stated in Chapter II, each compartment of the compartmented culture contains a completely separate fluid environment. This restriction of bulk fluid flow allows experimental procedures to take place in any one or two of the compartments while allowing the non-treated compartment(s)

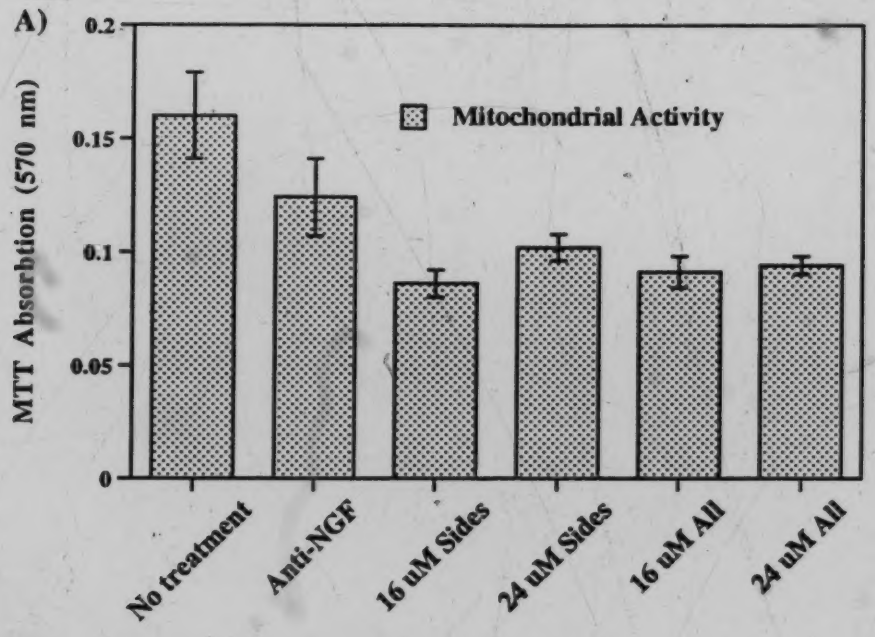
to remain as a control. The possibility of treating one segment of the neurons, such as the distal axons only, and investigating the results of this treatment on the other segment, like the cell bodies and proximal axons, are also possible with the compartmented culture. This type of examination suggests that the actions of the toxic peptide may be localized to either distal axons only or to the cell bodies/proximal axons. The latter type of analysis was performed using the compartmented culture system when apoE(141 - 149)$^2$, apoE(61 - 69)$^2$, apoE3(1 - 183), apoE4(1 - 183), or anti-NGF antibody were added to either the side distal axon-containing compartments or the centre cell body/proximal axon-containing compartments.

Concentration studies were done using a constant concentration of 24 nM anti-NGF antibody, and an increasing concentration of 16 - 32 μM of apoE(141 - 149)$^2$ on either the side compartments or the centre compartment only. An assessment of mitochondrial activity was used as a measurement of cell viability using the MTT assay (See chapter II) (Fig. 10). The application of 16 μM or 24 μM apoE(141 - 149)$^2$ on the distal axon-containing-compartments produced a significant decrease (P= 0.001) in mitochondrial function (Fig. 10A). No effect could be seen in the centre cell body/proximal axon-containing compartment when 24 μM apoE(141 - 149)$^2$ was added (P= 0.2) (Fig. 10B). When the centre compartment only was exposed to various concentrations of the synthetic tandem repeat peptide no effects were observed on mitochondrial function in either the cell bodies/proximal axons or the distal axons (P= 0.9)

Figure 10. Mitochondrial Activity Measured by MTT Assay of the Effects of Synthetic Tandem Repeat Peptide ApoE(141 - 149)$^2$ in Either Distal Axon-containing Compartments or All Three Compartments of the Compartmented Culture

Mitochondrial activity measured using an enzymatic MTT assay (Promega, Madison, WI) at an absorbance of 570 nm after treatments of apoE(141 - 149)$^2$ or anti-NGF antibody in either side compartments or all three compartments of the compartmented culture. Control group received 5 ng/ml NGF in side compartments, anti-NGF antibody, 24 nM, applied to side compartments only, and apoE(141 - 149)$^2$, 16 or 24 μM, applied to either side compartments only or all three compartments. A) Mitochondrial activity was measured in side compartments only and shows a decrease in activity in sides. B) Mitochondrial activity was measured in centre compartment only and shows no apparent effect on mitochondrial activity in the centre compartment when all three compartments are treated with 16 or 24 μM apoE(141 - 149)$^2$ . Results shown represent 3 dishes/treatment and are representative of three separate experiments.

P16 (P - P) = 16 μM apoE(141 - 149)$^2$ supplied to side compartments only
P24 (P - P) = 24 μM apoE(141 - 149)$^2$ supplied to side compartments only
P16 (PPP) = 16 μM apoE(141 - 149)$^2$ supplied to all three compartments
P24 (PPP) = 24 μM apoE(141 - 149)$^2$ supplied to all three compartments .


A)
MTT Absorbtion (570 nm)
0.2
0.15
0.1
0.05
0
Mitochondrial Activity
No treatment
Anti-NGF
16 uM Sides
24 uM Sides
16 uM All
24 uM All

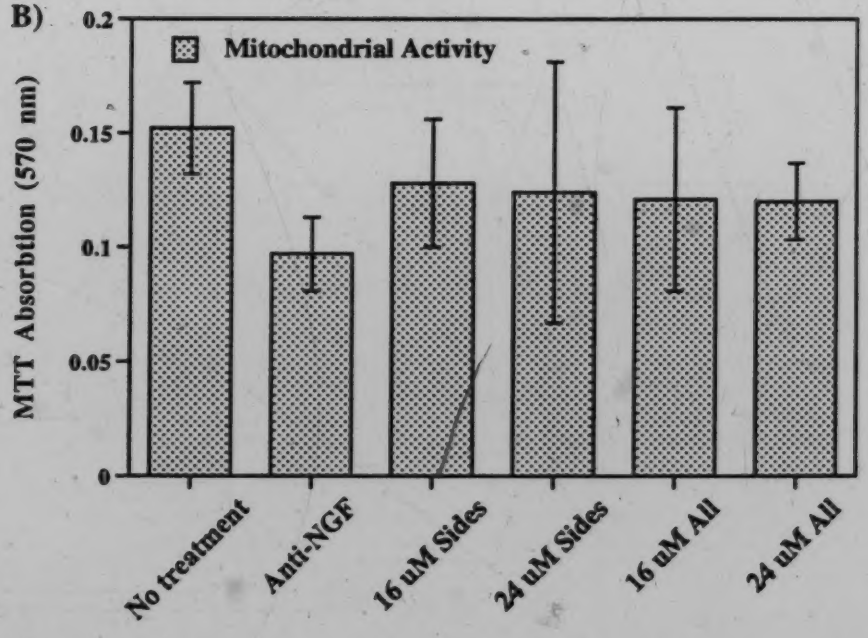
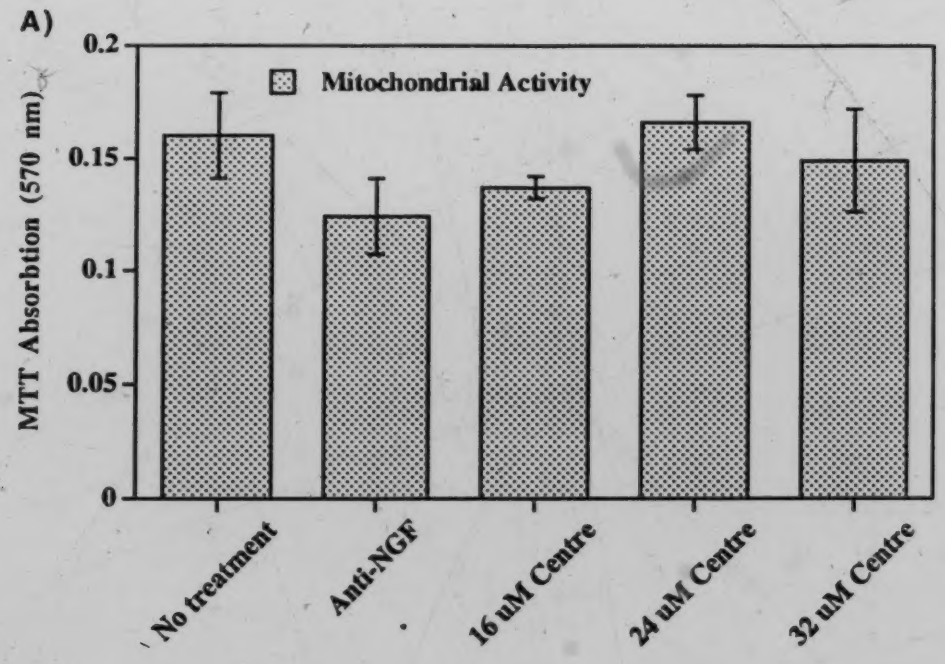
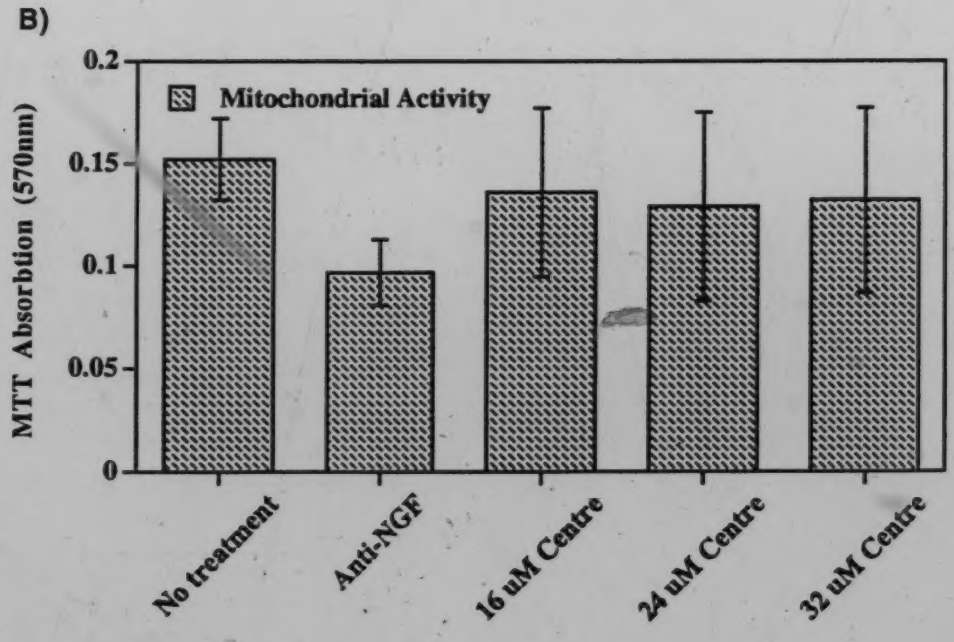

B)
MTT Absorbtion (570 nm)
0.2
0.15
0.1
0.05
0
Mitochondrial Activity
No treatment
Anti-NGF
16 uM Sides
24 uM Sides
16 uM All
24 uM All

Figure 11. Mitochondrial Activity Measured by MTT Assay of the Effects of Synthetic Tandem Repeat Peptide ApoE(141 - 149)$^2$ in the Cell body/proximal Axon-containing Compartment of the Compartmented Culture

Mitochondrial activity measured using an enzymatic MTT assay (Promega, Madison, WI) at an absorbance of 570 nm after treatments of apoE(141 - 149)$^2$ to the centre, cell body/proximal axon-containing compartment of the compartmented culture. Control group received 5 ng/ml NGF only in side compartments, 24 nM anti-NGF anti-body applied to side compartments only, and 16, 24, or 32 μM apoE(141 - 149)$^2$ applied to centre compartment only of the compartmented culture, A) mitochondrial activity measured in side compartments shows no significant difference in activity at 16, 24, or 32 μM apoE(141 - 149)$^2$ in the side compartments, B) mitochondrial activity measured in centre compartment shows no significant difference in activity at 16, 24, or 32 μM apoE(141 - 149)$^2$ in the centre compartment. Results shown represent 3 dishes/treatment and are representative of three separate experiments.

P16 (- P -) = 16 μM apoE(141 - 149)$^2$ supplied to centre compartment only
P24 (- P -) = 24 μM apoE(141 - 149)$^2$ supplied to centre compartment only
P32 (- P -) = 32 μM apoE(141 - 149)$^2$ supplied to centre compartment only


A)
MTT Absorbtion (570 nm)
0.2
0.15
0.1
0.05
0
Mitochondrial Activity
No treatment
Anti-NGF
16 uM Centre
24 uM Centre
32 uM Centre


B)
MTT Absorbtion (570nm)
0.2
0.15
0.1
0.05
0
Mitochondrial Activity
No treatment
Anti-NGF
16 uM Centre
24 uM Centre
32 uM Centre

(Fig. 11). Furthermore, a concentration as high as 32 μM had no effect (P= 0.4) on these two-week-old cultures when applied to the centre cell body/proximal axon-containing compartment (Fig. 11).

## III.3 MASS CULTURES OF PRIMARY RAT SYMPATHETIC NEURONS

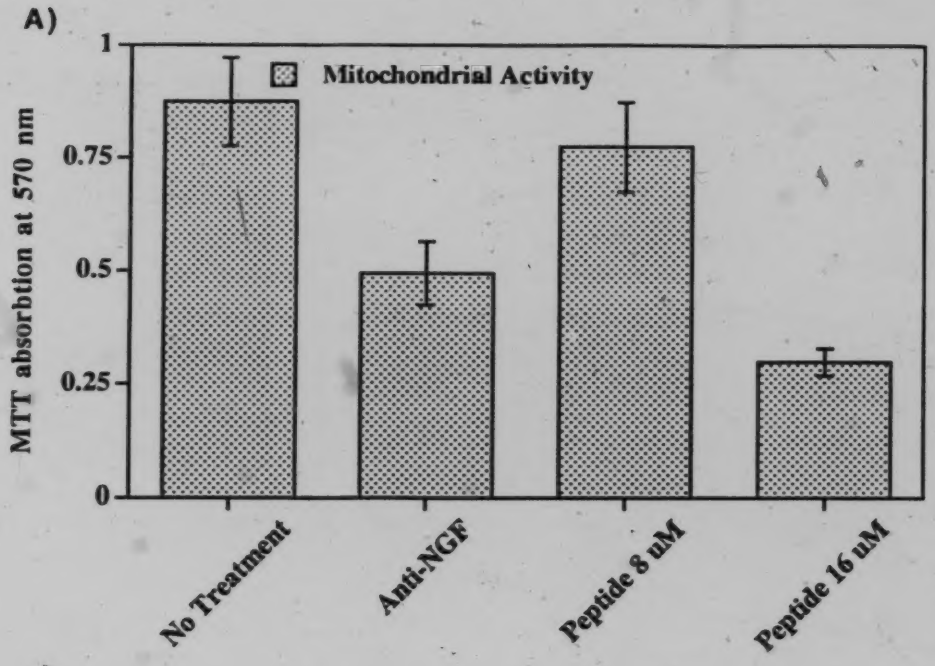
Whether mass cultures of primary rat sympathetic neurons would undergo the same consequence when treated with 8 μM apoE(141 - 149)$^2$ as mass cultures of chicken sympathetic neurons and rat hippocampal neurons, used by Tolar and coworkers (1997), was examined next.

As with the compartmented culture system, trypsinized and mechanically dissociated rat superior cervical ganglia were plated on rat tail collagen-coated 24 well plates at approximately 1.0 ganglia/well. Mass cultures were given all the necessary nutrients for optimal growth. One-week-old mass cultures were exposed to one of the following: a) 10 ng/ml NGF, b) 24 nM anti-NGF antibody, c) 8 μM apoE(141 - 149)$^2$ or, d) 16 μM apoE(141 - 149)$^2$ for 24 or 48 hours (Fig. 12, 13, 14, 15). Unlike previous studies (Crutcher *et al.* 1994, 1998, Tolar *et al.* 1997) with mass cultures of chicken sympathetic neurons, 8μM apoE(141 - 149)$^2$ tandem repeat peptide had no significant effect on these mass cultures of rat sympathetic neurons at either 24 or 48 h exposure (Fig. 12). However, both anti-NGF antibody and 16 μM apoE(141 - 149)$^2$ displayed a significant decrease in mitochondrial function after 24 h treatment (Fig. 12A).

Figure 12. Mitochondrial Activity Measured after Treatment with Anti-NGF or ApoE(141 - 149)$^2$ Peptides for 24 or 48 hours on Mass Cultures of Rat Sympathetic Neurons

Mitochondrial activity measured using an enzymatic MTT assay (Promega, Madison, WI) at an absorbance of 570 nm after 24 or 48 hours treatment on mass cultures of rat sympathetic neurons. Control group received 5 ng/ml NGF only, anti-NGF group received 24 nM anti-NGF antibody, and the synthetic tandem repeat peptide group received 8 - 16 μM apoE(141 - 149)$^2$. A) Mitochondrial activity measured after 24h shows a significant decrease in activity when anti-NGF antibody or 16 μM apoE(141 - 149)$^2$ were supplied to the mass cultures but when 8 μM apoE(141 - 149)$^2$ was supplied to mass cultures no apparent change in mitochondrial activity is noted, B) Mitochondrial activity measured after 48 h shows a significant decrease in activity of both the anti-NGF and 16 μM apoE(141 - 149)$^2$ treatment groups. Results represent 3 wells/treatment and are representative of three separate experiments.

Peptide = apoE(141 - 149)$^2$


A)
Mitochondrial Activity
MTT absorbtion at 570 nm
1
0.75
0.5
0.25
0
No Treatment
Anti-NGF
Peptide 8 uM
Peptide 16 uM

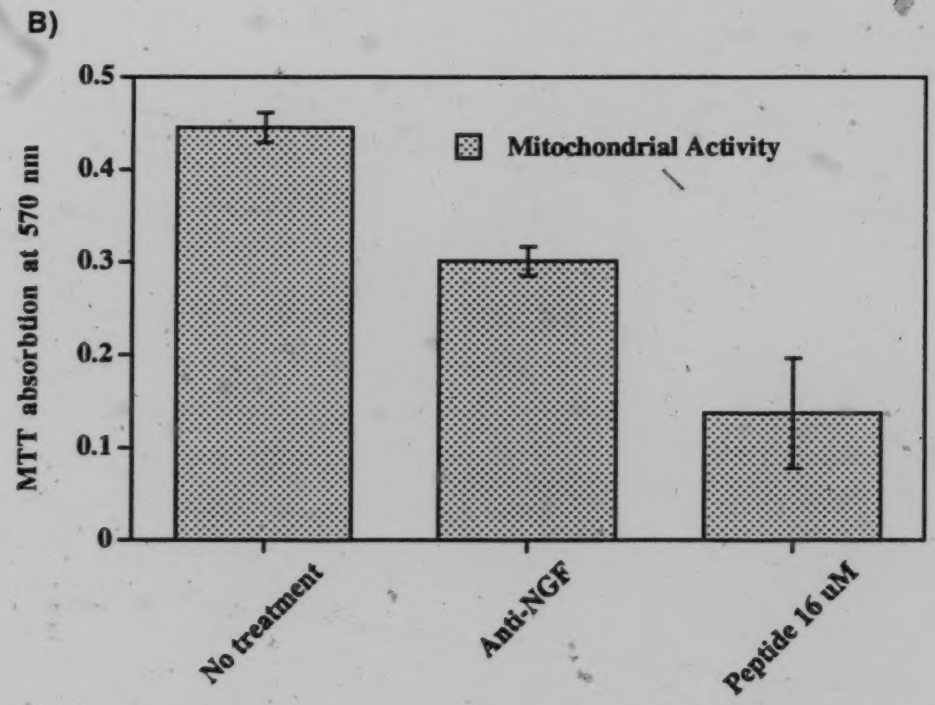

B)
Mitochondrial Activity
MTT absorbtion at 570 nm
0.5
0.4
0.3
0.2
0.1
0
No treatment
Anti-NGF
Peptide 16 uM

Figure 13. Nerve Growth Factor Treatment of Mass Cultures of Rat Sympathetic Neurons Shows No Apparent Morphological Changes After 24h Treatment

Phase contrast micrograph of mass cultures of rat sympathetic neurons before and after treatment with 5 ng/ml NGF. A) Representative neurons of mass cultures before treatment, B) Identical neurons of mass cultures 24h after treatment with NGF only. Results shown are representative of 3 wells/treatment and > 3 separate experiments Magnification = 2,000 X.

A

B

Figure 14. Anti-NGF Antibody Treatment of Mass Cultures of Rat Sympathetic Neurons Shows Neurite Beading After 24h Treatment

Phase contrast micrograph of mass cultures of rat sympathetic neurons before and after treatment with 24 nM anti-NGF antibody. A) Representative neurons of mass culture before treatment, B) Representative neurons of mass cultures showing neurite beading after 24h treatment with anti-NGF antibody. Results shown representative of 2 separate experiments. Magnification = 2,000 X.

A

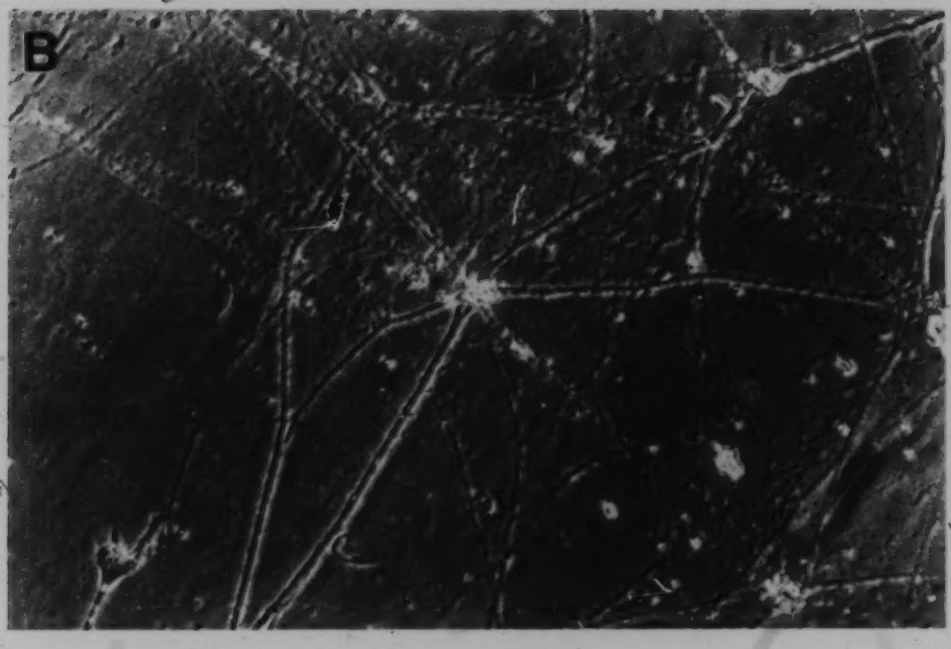
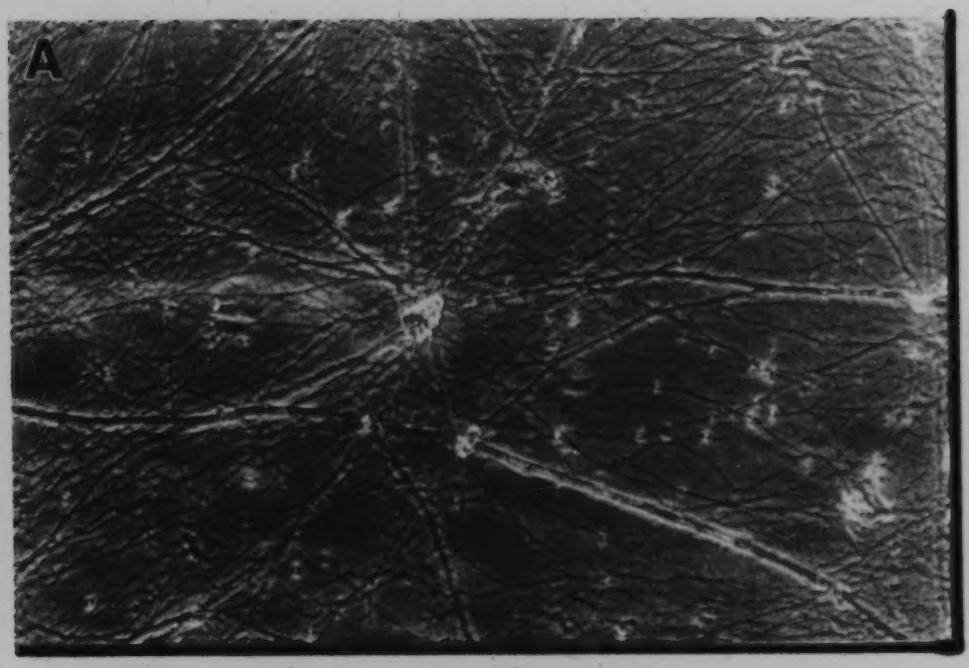
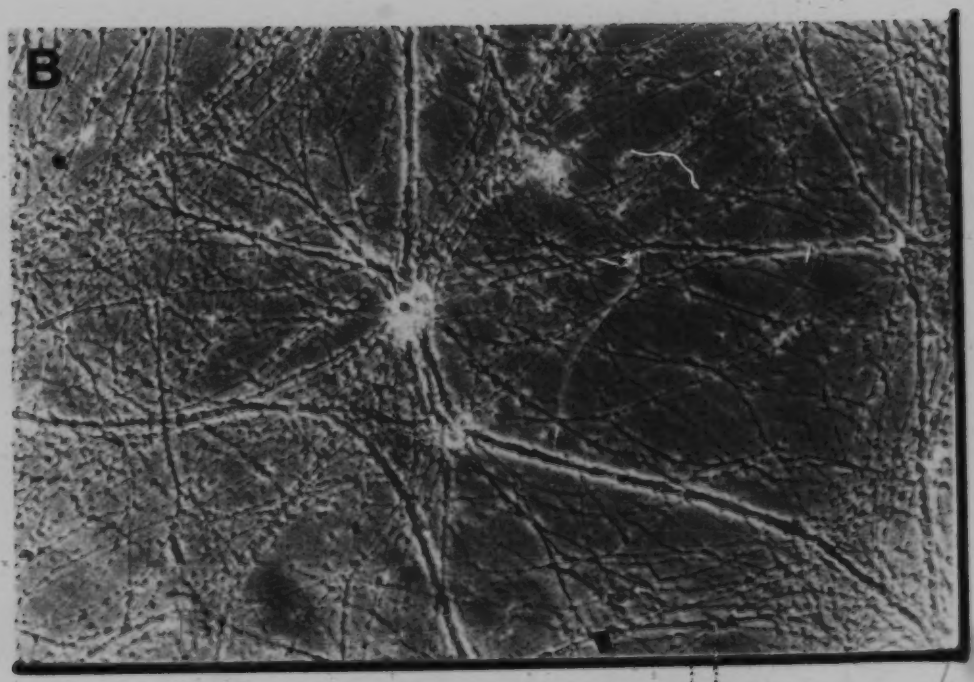

B

Figure 15. ApoE(141 - 149)$^2$ Treatment on Mass Cultures of Rat Sympathetic Neurons Shows No Morphological Changes After 24h Treatment

Phase contrast micrograph of mass cultures of rat sympathetic neurons before and after treatment with synthetic apoE(141 - 149)$^2$ tandem repeat peptide. A) Representative neurons of mass cultures before treatment, B) Identical neurons of mass cultures after 24h treatment shows no morphological changes. Results shown representative of > 3 separate experiments. Magnification = 2,000 X.


A


B

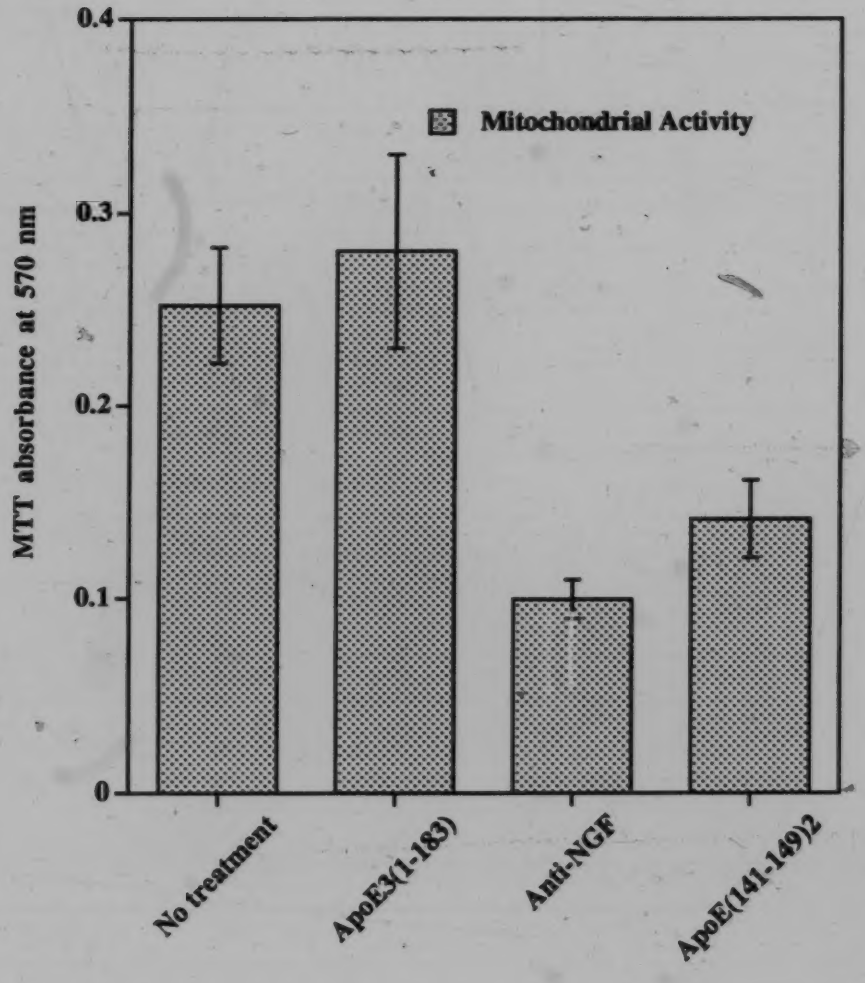
After a 48 h exposure to either 24 nM anti-NGF (P= 0.01) or 16 μM of the apoE(141-149)$^2$ tandem repeat peptide (P= 0.01), a significant decrease in mitochondrial activity was noted between the peptide and the control group. The trend seen of decreased mitochondrial activity, when using 16 μM apoE(141 - 149)$^2$, is consistent between both the 24 h and the 48 h time periods (Fig. 12 A,B)

N-terminal apoE3(1 - 183) is similar to the apoE3 22 kDa fragment seen in the CSF of both control and AD subjects (Tolar *et al.* 1997). Previous studies done with both apoE3 and apoE4 22 kDa thrombin cleavage fragments showed two very interesting results: 1) the apoE3 fragment caused a decrease in cell viability when chicken sympathetic neurons were treated with 1 μM, and 2) those cultures treated with only 0.25 μM apoE4 22 kDa fragment displayed almost total elimination of viable cells (Crutcher *et al.* 1998).

We next examined the effects of apoE3(1 - 183) on mass cultures of rat sympathetic neurons. Cell viability was again measured using the MTT assay on one-week old mass cultures treated with 24 nM anti-NGF antibody, 16μM apoE(141 - 149)$^2$, or 30μM N-terminal apoE3(1 - 183). When N-terminal apoE3(1 - 183) was added to the mass cultures of rat sympathetic neurons no significant effect (P= 0.6) on the viability could be detected (Fig. 16). This is surprising in light of the fact that only 1 μM of apoE3 22 kDa fragment decreased cell viability by at least 50% (Crutcher *et al.* 1998). Both anti-NGF (P= 0.001) and the synthetic peptide (P= 0.001) displayed significant

Figure 16. Mitochondrial Activity Measured After Treatment of Mass Cultures of Rat Sympathetic Neurons with ApoE3(1 - 183), Anti-NGF Antibody, or ApoE(141 - 149)$^2$

Mass cultures were prepared as described in Methods and mitochondrial activity measured using an enzymatic MTT assay (Promega, Madison, WI) at an absorbance of 570 nm after 24 h treatment. Control group received 5 ng/ml NGF only, apoE3(1 - 183) [30 μM], anti-NGF antibody [24 nM], and apoE(141 - 149)$^2$ [16 μM]. Both the control and apoE3(1 - 183) treatment groups displayed no significant difference in mitochondrial activity but the anti-NGF and apoE(141 - 149)$^2$ treatment groups showed a significant decrease in activity after 24h. Results represent 3 wells/treatment and are representative of three separate experiments.


Mitochondrial Activity
MTT absorbance at 570 nm
0.4
0.3
0.2
0.1
0
No treatment
ApoE3(1-183)
Anti-NGF
ApoE(141-149)2

decreases in cell viability when the mass cultures were exposed to both treatments for 24 h (Fig. 16). Older cultures may have a protective effect on treatments with toxic peptides, at the lower concentration of 8 μM, but when the concentration of apoE(141 - 149)$^2$ was increased to 16 μM this protective effect may have been nullified.

## III.4 AXOTOMY AND MEASUREMENT OF NEURITE REGENERATION

As mentioned previously, a distinct advantage of the compartmented culture system is the ability of the researcher to axotomize the distal axons while leaving the cell bodies and proximal axons intact. Once axotomy has been performed, regeneration of neurites can be measured in the side compartments after addition of various treatments to either the side or centre compartments.

Two week old cultures were axotomized and the neurites allowed to regenerate for 4 days. ApoE(141 - 149)$^2$ [16 μM] plus 10 ng/ml NGF, apoE(61 - 69)$^2$ [16 μM] plus 10 ng/ml NGF or 10 ng/ml NGF only (control) were added on day 4 post-axotomy and neurite regeneration was measured for two days following treatment (Fig. 17). Twenty-four hours after treatment both the control group and the apoE(61 - 69)$^2$ tandem repeat peptide (control peptide) had no effect on neurite regeneration, but the apoE(141 - 149)$^2$ tandem repeat peptide completely destroyed the neurites (P= 0.001) (Fig. 17 & Fig. 18A). On day 2

Figure 17. Measurement of regeneration after axotomy and treatment with either apoE(61 - 69)$^2$ or apoE(141 - 149)$^2$

Four days after axotomy the distal axon-containing compartments were exposed to various treatments and neurite regeneration measured for two days. Control group received 10 ng/ml NGF, synthetic tandem repeat peptide apoE(61 - 69)$^2$ [16 μM], and synthetic tandem repeat peptide apoE(141 - 149)$^2$ [16 μM].

Neurite regeneration was measured in 3 cultures/treatment after axotomy for day 1 following treatment. One dish/treatment was removed from both the control group and the apoE(61 - 69)$^2$ treatment group because the neurites had reached the outermost limit of the side compartment. Means ± SD are plotted of the following: I) 15 tracks for no treatment group on 1 and 10 tracks on day 2, II) 19 tracks for anti-NGF for days 1 and 2 following treatment, III) 35 tracks for apoE(61 - 69)$^2$ on day 1 and 29 tracks for day 2, and IV) 27 tracks for apoE(141 - 149)$^2$ for days 1 and 2 after treatments added. Results shown are representative of two individual experiments.

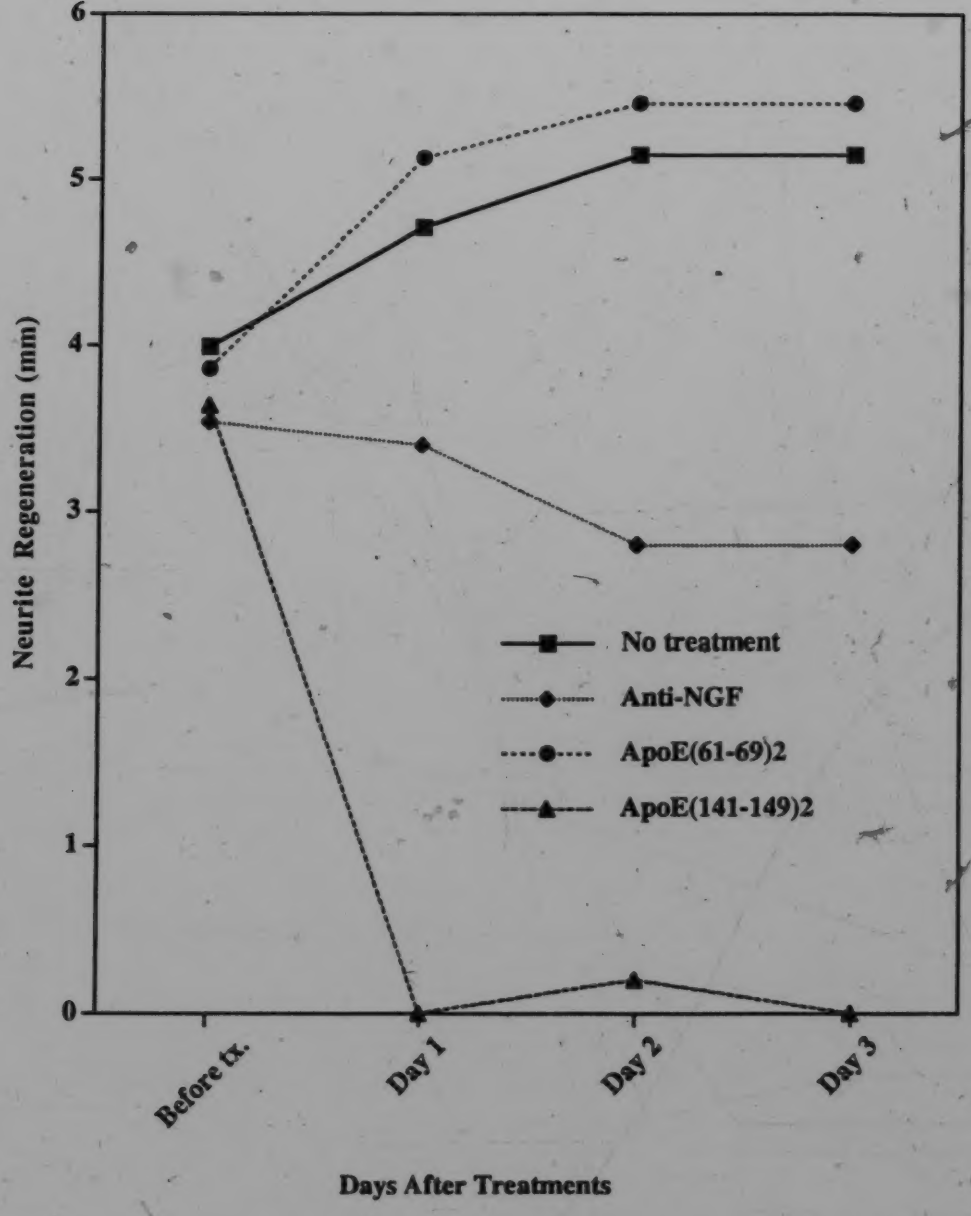
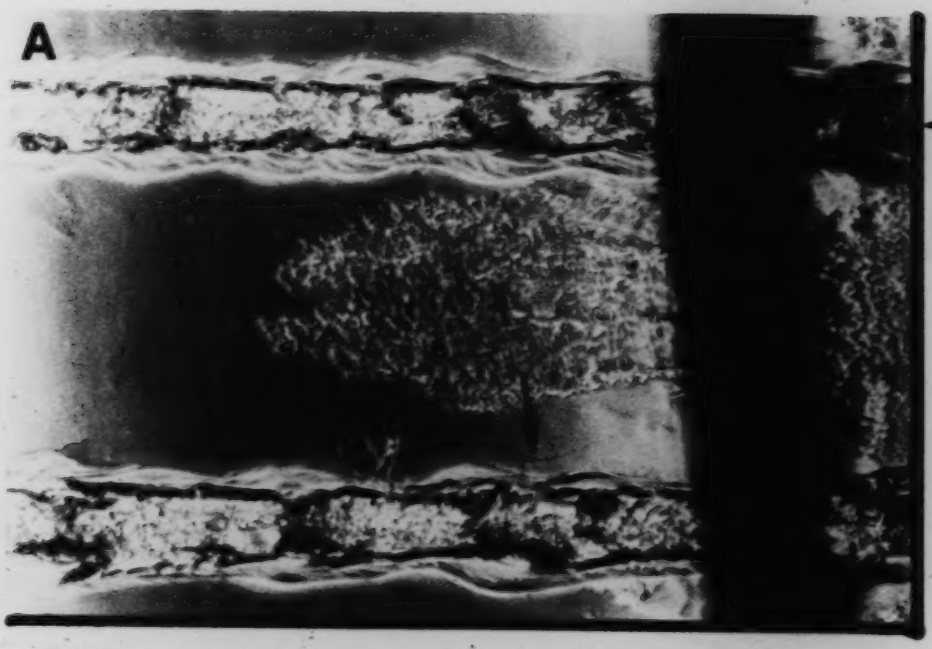

Neurite Regeneration (mm)
6
5
4
3
2
1
0
No treatment
Anti-NGF
ApoE(61-69)2
ApoE(141-149)2
Before tx.
Day 1
Day 2
Day 3
Days After Treatments

Figure 18. ApoE(141 - 149)$^2$ Treatment to the Side Compartments Completely Destroys the Distal Axons While Treatment of ApoE(141 - 149)$^2$ to the Cell Bodies/Proximal Axons in the Centre Compartment Does Not Appear to Cause Either Degeneration of Proximal Axons or Cell Death

Phase contrast micrograph of rat sympathetic neurons on a single track after 24h treatment with apoE(141 - 149)$^2$. Panels are ~ 1.0 mm long and scratches (see arrowhead) can be seen in the substratum on top and bottom of both micrographs. A) Left hand side compartment containing degenerated distal axons after 24h treatment of apoE(141 - 149)$^2$ [16 μM]. New neurite sprouting (see arrow) seen after 48 h treatment. Dark vertical shadow indicates line of grease. B) Intact cell bodies and proximal axons in centre compartment after exposure to 16 μM apoE(141 - 149)$^2$ for 24 h. Shown is micrograph representative of > 20 dishes. Magnification = 2,000 X


A

B

post treatment the dishes containing apoE(141-149)$^2$ tandem repeat peptides began to regenerate their neurites (Fig. 18A, see arrow). Because of this regeneration, the side compartments of these specific cultures were washed and exposed to fresh apoE(141 - 149)$^2$ [16 μM]. The following day the neurites in these dishes were again totally destroyed (Results not shown).

This destruction and regeneration suggests two possible explanations: 1) apoE(141 - 149)$^2$ may also need to be exposed to the proximal axons, but not cell bodies, in order to inhibit neurite regeneration altogether, or 2) a continual supply of apoE peptides may be necessary to overcome any protective effect of the degenerating axons. Possible protective mechanisms may be release of proteases or peptidases that cleave the peptide bonds or some other protein/enzyme that may bind to the peptides and prevent any further destructive effects.

Addition of apoE(141 - 149)$^2$ peptides to axotomized cultures on day 4 post axotomy again displayed complete degeneration of the neurites 24 h after treatments were added (Fig. 19 & Fig. 20). Anti-NGF antibody treatment showed a decrease in neurite extension that appeared to level off after three days treatment (Fig. 19). The control peptide, apoE(61 - 69)$^2$ had no apparent effect on neurite regeneration (Fig. 19). On day 2, after treatment, the right hand side (RHS) of four apoE(141 - 149)$^2$ treated cultures were washed and fresh media containing apoE(141 - 149)$^2$ [16 μM] was again added. The left hand sides (LHS) of all four dishes were untouched to see if neurite extension would

Figure 19. Measurement of regeneration after axotomy and treatment with anti-NGF antibody, apoE(61 - 69)$^2$, or apoE(141 - 149)$^2$

Four days after axotomy distal axon-containing compartments were exposed to various treatments and neurite regeneration measured for three days after treatments applied. Control group received 10 ng/ml NGF, anti-NGF [24 nM], synthetic tandem repeat peptide apoE(61 - 69)$^2$ [16 μM], synthetic tandem repeat peptide apoE(141 - 149)$^2$ [16 μM].

Neurite regeneration was measured in 3 cultures/treatment after axotomy. One dish/treatment was removed from both the no treatment group and the apoE(61 - 69)$^2$ group because the neurites had reached the outermost limit of the side compartment. Means are plotted of the following: I) 37 tracks for NGF only on day 1 and 27 tracks on day 2 following treatment, II) 27 tracks for anti-NGF on days 1 and 2 following treatment, III) 34 tracks for apoE(61 - 69)$^2$ on day 1 following treatment and 24 tracks on day 2 following treatment, and IV) 22 tracks for apoE(141 - 149)$^2$ for days 2 and 3 after treatments added. Results shown are representative of > 3 separate experiments.

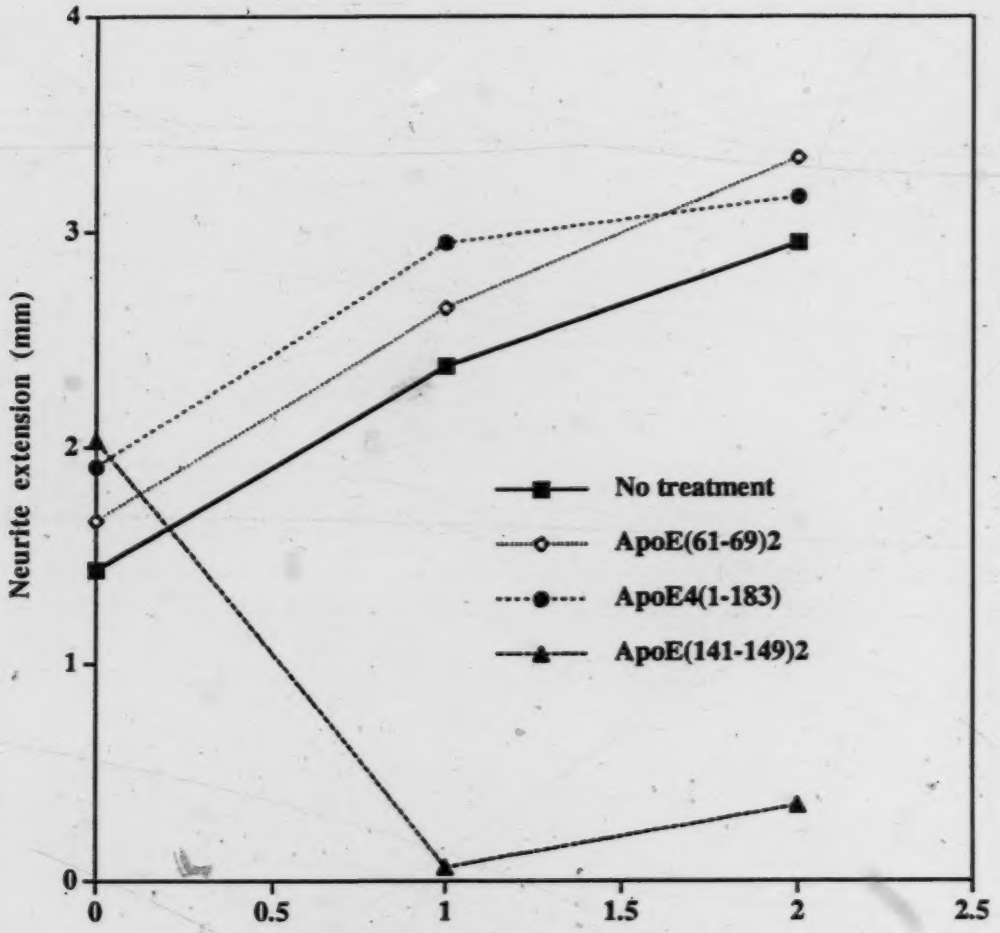
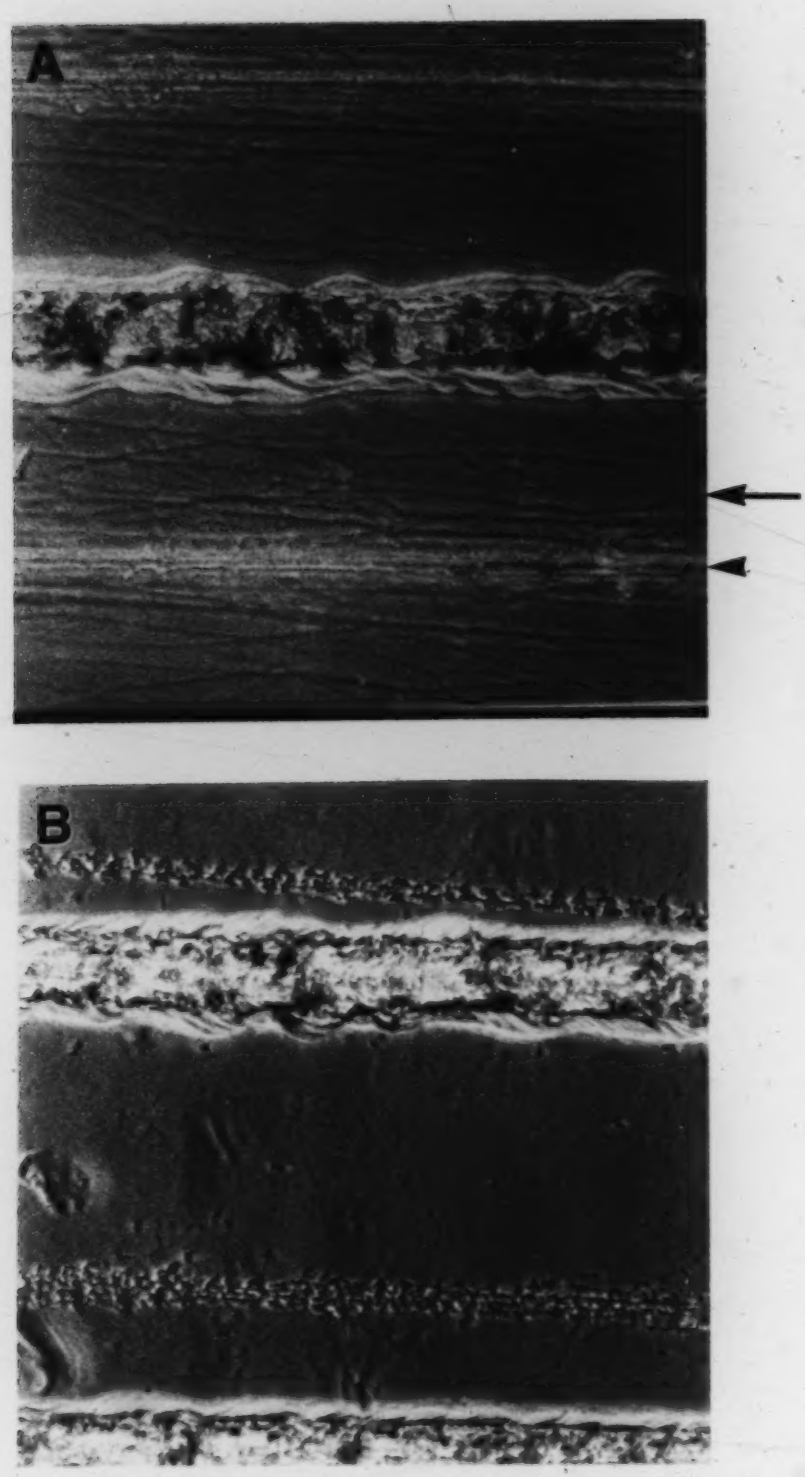

Neurite extension (mm)
4
3
2
1
0
0
0.5
1
1.5
2
2.5
No treatment
ApoE(61-69)2
ApoE4(1-183)
ApoE(141-149)2
Days after treatments added

Figure 20. Treatment of ApoE(141 - 149)$^2$ to Side Compartments Only of the Compartmented Culture Completely Destroys Distal Axons After 25.5h

Phase contrast micrograph of rat sympathetic neurons on a single track after 25.5h treatment with 16 μM apoE(141 - 149)$^2$. Panels are ~ 1.0 mm long and a single scratch in the substratum can be seen in the top panel and two scratches can be seen in the bottom panel. A) Right hand side compartment containing axonal cord (see arrowhead) and fine branching (see arrow) can be seen before treatment, B) Degenerated axons in right hand compartment seen after treatment with apoE(141 - 149)$^2$ for 25.5h. Results shown are representative of > 3 separate experiments. Magnification = 2,000 X.


A
B

continue in the LHS. Again the RHS of all four dishes displayed complete neurite degeneration but, all four LHS compartments showed continued neurite extension, albeit at a slower rate of growth (< 1 mm). (Results not shown) While normally neurite regeneration occurs at ~ 1 mm/day, it may be possible that once the apoE(141 - 149)$^2$ peptides are no longer able to inhibit neurite extension, and no new peptides are supplied to the cultures, the neuronal cells may simply need time to overcome these devastating effects and regenerate at their normal rates.

Axotomized cultures were treated with 16 μM apoE(61 - 69)$^2$, 16 μM apoE(141 - 149)$^2$, or 30 μM apoE4(1 - 183). Neurite degeneration and subsequent regeneration was seen with the apoE(141 - 149)$^2$ treated cultures (Fig. 21 & 22). ApoE4(1 - 183) had no apparent effect on neurite regeneration 20.5 h after treatments (Fig. 21). Some granulation can be seen by 20.5 h (Fig. 22B) and by 43.5 h exposure, the granulation had increased considerably (Fig. 22C). This increase in granulation may be the reason why neurite extension began to decrease by the second day after apoE4(1 - 183) treatment was added (Fig. 21). Axotomized cultures were treated with 16 μM apoE(61 - 69)$^2$, 16 μM apoE(141 - 149)$^2$, 30 μM apoE3(1 - 183), 30 μM apoE4(1 - 183), or 10 ng/ml NGF (control group) in either sides and centre compartments or side compartments only. Treatments were added on day 2 post axotomy and by day 3 total neurite destruction was seen in those dishes treated with apoE(141 - 149)$^2$ (P= 0.001) (Fig. 23, 24). ApoE4(1 - 183) did not appear to inhibit neurite extension when treatment was applied to side compartments only (P= 0.7), but

Figure 21. Measurement of regeneration after axotomy and treatment with apoE(61 - 69)$^2$, apoE(141 - 149)$^2$, or apoE4(1 - 183)

Two days after axotomy distal axon-containing compartments were exposed to various treatments and neurite regeneration measured for two days. Control group 10 ng/ml NGF, apoE(61 - 69)$^2$ [16 μM], synthetic tandem repeat peptide apoE(141 - 149)$^2$ [16 μM], lipid-free apoE4(1 - 183) [30 μM].

Neurite regeneration was measured in 3 cultures/treatment after axotomy. Means are plotted of the following: I) 28 tracks for NGF only, II) 40 tracks for apoE(61 - 69)$^2$, III) 15 tracks for apoE(141 - 149)$^2$, and IV) 20 tracks for apoE4(1 - 183) measurements taken for two days. Results shown are representative of > 3 separate experiments. Magnification = 2,000 X.

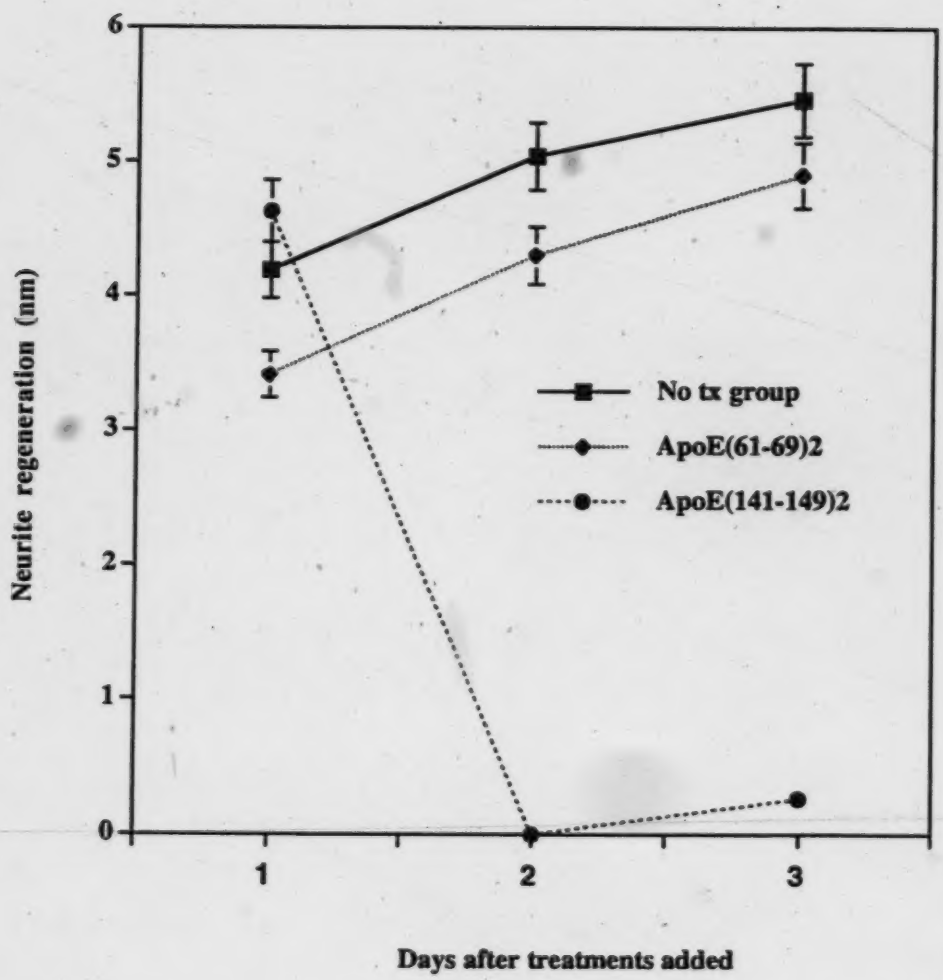

6
5
4
3
2
1
0
Neurite regeneration (nm)
1
2
3
Days after treatments added
No tx group
ApoE(61-69)2
ApoE(141-149)2

Figure 22. Lipid-free ApoE4(1 - 183) Treatment to the Side Compartments Shows No Apparent Change in Morphology After 20.5h Treatment But an Increase in Granulation Can Be Seen After 43.5h Treatment

Phase contrast micrographs of rat sympathetic neurons on a single track before and after 20.5 and 43.5h treatments with lipid-free apoE4(1 - 183). Panels are ~ 1.0 mm. A) Right hand side distal axon-containing compartment before treatment, B) Right hand side after 20.5 h treatment with some granulation seen, and, C) Right hand side after 43.5h treatment with increased granulation seen. Micrographs representative of 4 dishes/treatment and represent duplicate experiments. Magnification = 2,000 X

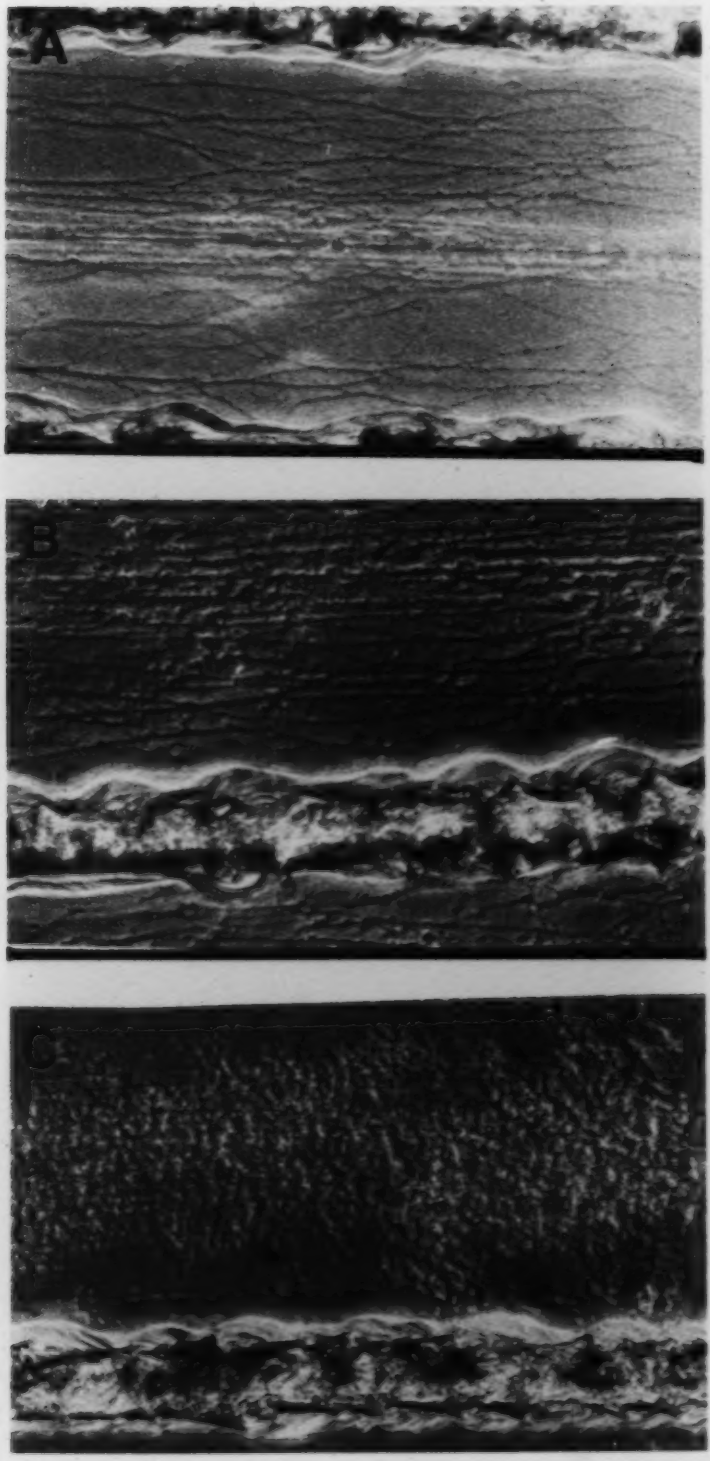

A
B
C

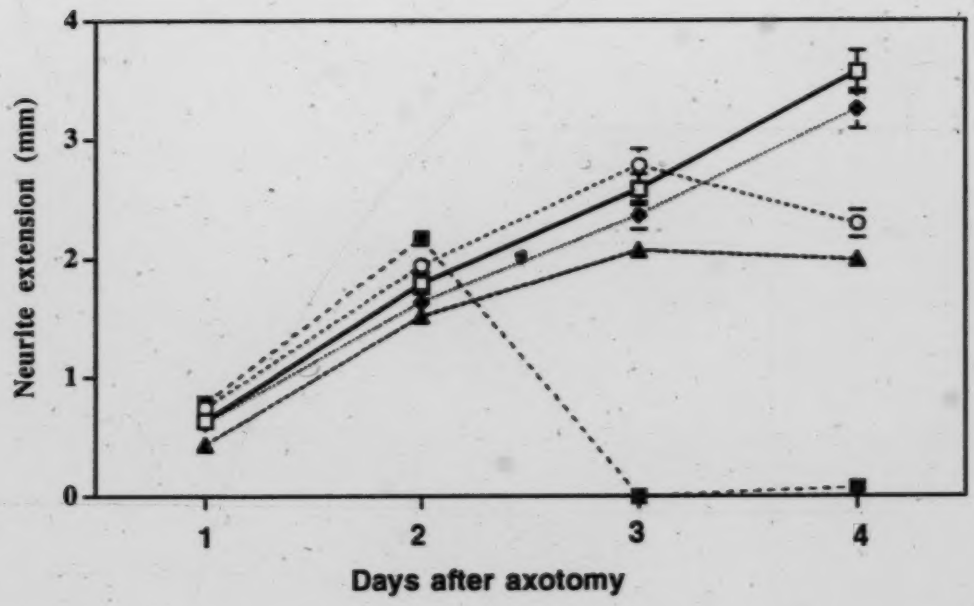
Figure 23. Measurement of Regeneration After Axotomy and Treatment with ApoE(61 - 69)$^2$, ApoE(141 - 149)$^2$, ApoE3( 1 - 183), or ApoE4(1 - 183) to Side Compartments Only or to All Three Compartments of the Compartmented Culture

Two days after axotomy either the centre cell body/proximal axon-containing compartment or all three compartments of the compartmented culture were supplied with various treatments and neurite regeneration measured for two days. Control group 10 ng/ml NGF, apoE(61 - 69)$^2$ [16μM], apoE(141 - 149)$^2$ [16μM] lipid-free apoE3(1 - 183) [30 μM], lipid-free apoE4(1 - 183) [16μM].

A) Treatments to all three compartments. Open boxes represent control group, filled diamonds represent treatment of apoE(61 - 69)$^2$, open circle represent lipid-free apoE3(1 - 183), filled triangles represent lipid-free apoE4(1 - 183), and filled boxes represent apoE(141 - 149)$^2$ treatment. B) Treatments to side compartments only. Open boxes represent control group, filled diamonds represent lipid-free apoE3(1 - 183), open circles represent lipid-free apoE4(1 - 183), and filled triangles represent apoE(141 - 149)$^2$

Panel A: I) 14 tracks for NGF only, II) 27 tracks for apoE(61 - 69)$^2$, III) 30 tracks for apoE(141 - 149)$^2$, IV) 30 tracks for lipid-free apoE3(1 - 183), and V) 29 tracks for lipid-free apoE491 - 183) for days 3 and 4 after treatments added. Panel B: I) 14 tracks for NGF only, II) 30 tracks for apoE(141 - 149)$^2$, III) 30 tracks for lipid-free apoE3(1 - 183), and IV) 19 tracks for lipid-free apoE4(1 - 183) for days 3 and 4 after treatments added. Neurite regeneration measured in 4 cultures/treatment after axotomy and are representative of three separate experiments.

A)


Neurite extension (mm)
0
1
2
3
4
1
2
3
4
Days after axotomy

B)

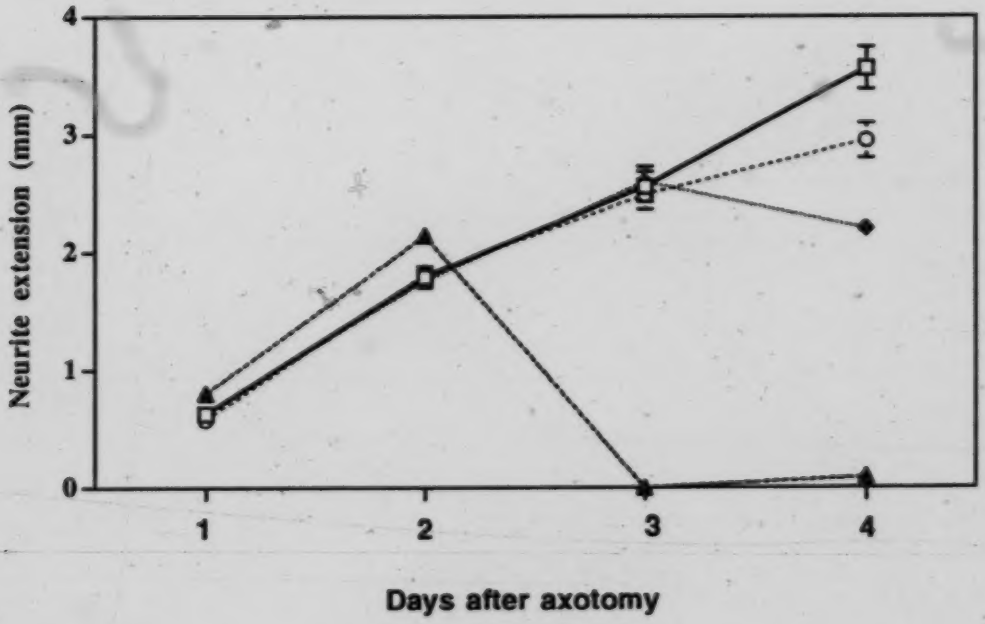
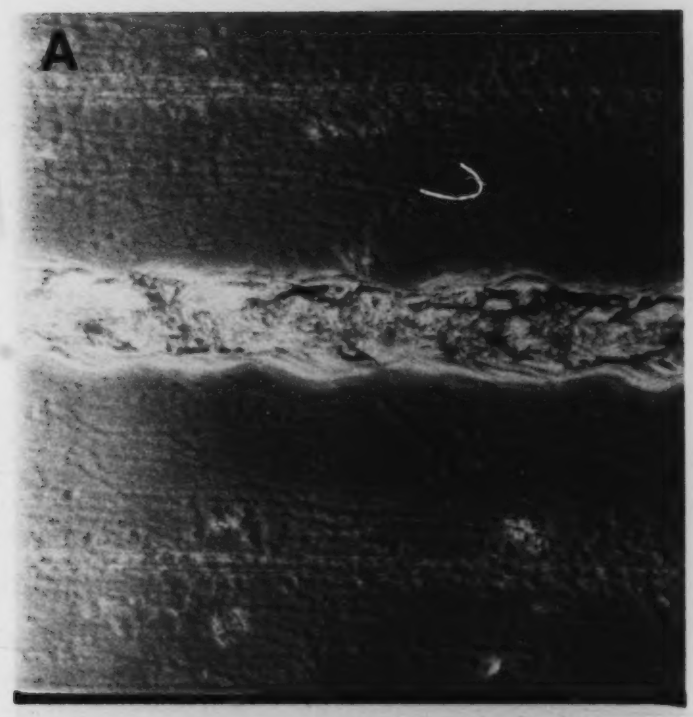
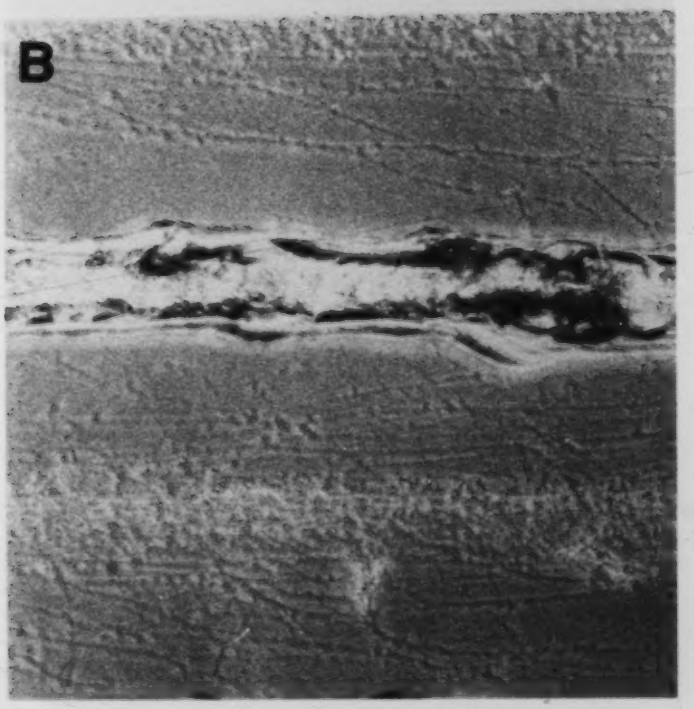

Neurite extension (mm)
0
1
2
3
4
1
2
3
4
Days after axotomy

Figure 24. ApoE(141 - 149)$^2$ Treatment on Centre Compartment Shows No Apparent Change in Morphology on Distal Axons After 24h Treatment

Phase contrast micrograph of rat sympathetic neurons on two tracks before and after treatment with synthetic apoE(141 - 149)$^2$ tandem repeat peptide in the centre cell body/proximal axon-containing compartment. Panels are ~ 1.0 mm long and a single scratch in the substratum can be seen in both panels A and B. A) Right hand side compartment containing axon cord and branching before treatment, B) Right hand side compartment containing axon cord and branching after treatment in centre compartment only shows no apparent change in morphology. Results are representative of three dishes/ treatment and > 3 separate experiments. Magnification = 2,000 X.

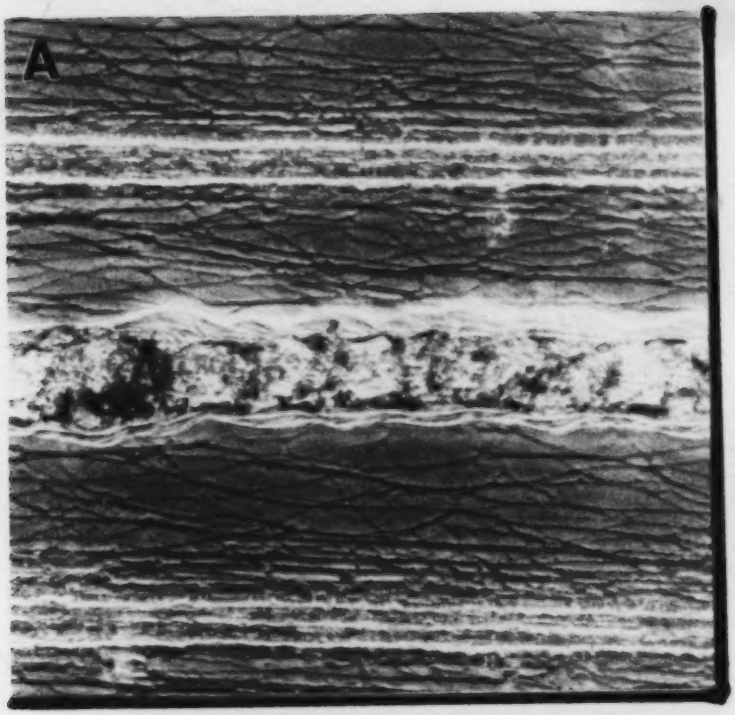

A


B

did appear to decrease after three days treatment when applied to all three compartments (P= 0.05) (Fig. 23). A slight decrease in neurite extension could be seen in cultures treated with apoE3(1 - 183) after 4 days in culture (Fig. 23), although overall gross appearance of the RHS compartment before and after treatment with 30 μM apoE3(1 - 183) was not significant (Fig. 25). The control peptide, apoE(61 - 69)$^2$, had no apparent effect on neurite regeneration (Figs. 17, 19, 21, 23). When treatments were applied to the centre, cell body/proximal axon-containing compartment none of the treatments had an effect on neurite regeneration (Figs. 23, 26, 27, 28). Phase micrographs of mass cultures treated with apoE3(1 - 183) (Fig. 29), apoE4(1 - 183) (Fig. 30), and bovine serum albumin (Fig. 31) were also produced and showed no apparent effect on morphology.

Figure 25. Treatment of Lipid-free ApoE3(1 - 183) to Side Compartments Has No Apparent Effect on Morphology on Distal Axons After 20.5h Treatment

Phase contrast micrograph of rat sympathetic neurons on two tracks before and after treatment with 30 μM lipid-free apoE3(1 - 183). Panels are ~ 1.0 mm long. A) Right hand side distal axon-containing compartment showing axonal cord and fine branching before treatment, B) Right hand side distal axon-containing compartment showing axonal cord and fine branching after 20.5h treatment. Results are representative of 4 dishes/treatment and two separate experiments. Magnification = 2,000 X.


A

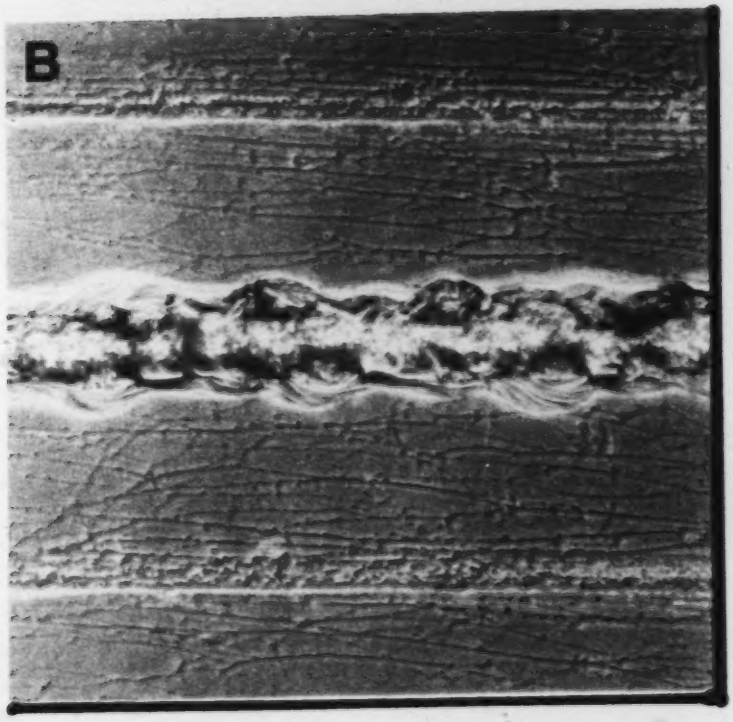
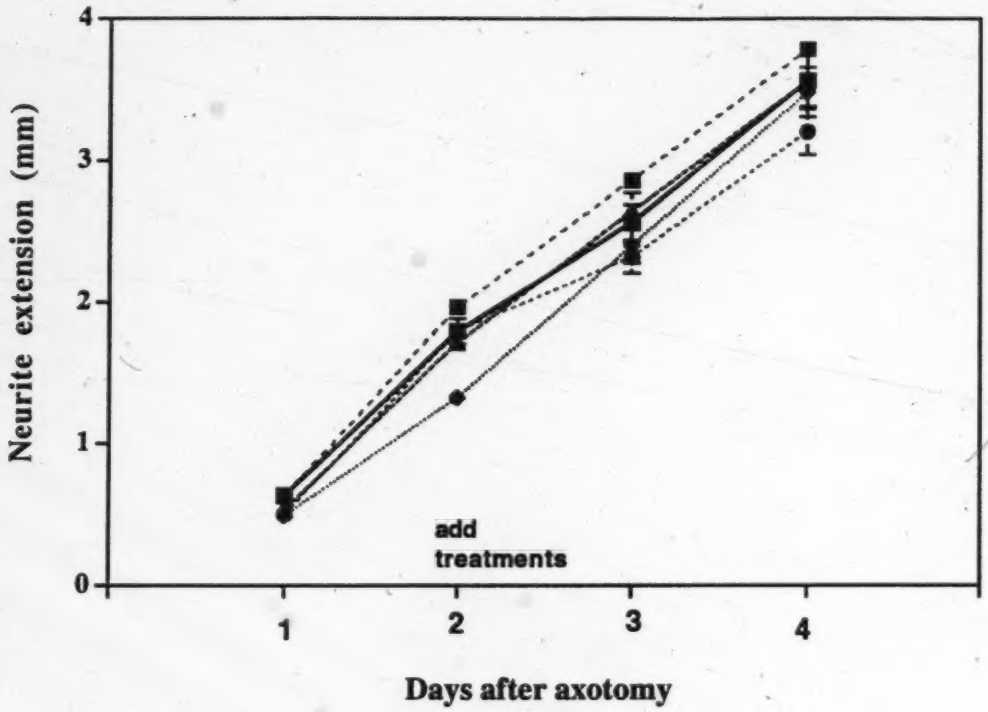

B

Figure 26. Measurement of Regeneration After Axotomy and Treatment with ApoE(61 - 69)$^2$, ApoE(141 - 149)$^2$, ApoE3(1 - 183), or ApoE4(1 - 183) to Centre Cell body/proximal Axon-containing Compartment Only

Two days after axotomy the centre cell body/proximal axon-containing compartments were supplied with various treatments and neurite regeneration measured for two days. Filled boxes represent base medium only, filled diamonds represent apoE(61 - 69)$^2$ [16 μM], filled circles represent lipid-free apoE3(1 - 183) [30 μM], filled triangles represent lipid-free apoE4(1 - 183) [30 μM], and filled boxes represent apoE(141 - 149)$^2$ [16 μM].

Neurite regeneration was measured in 4 cultures/treatment after axotomy. Means ± SD are plotted of the following: I) 14 tracks for NGF only, II) 29 tracks for apoE(61 - 69)$^2$, III) 30 tracks for apoE(141 - 149)$^2$, IV) 30 tracks for lipid-free apoE3(1 - 183), or V) 30 tracks for lipid-free apoE4(1 - 183) for days 3 and 4 after treatments added. Results shown are representative of two individual experiments.




no treatment

ApoE(61 - 69)2

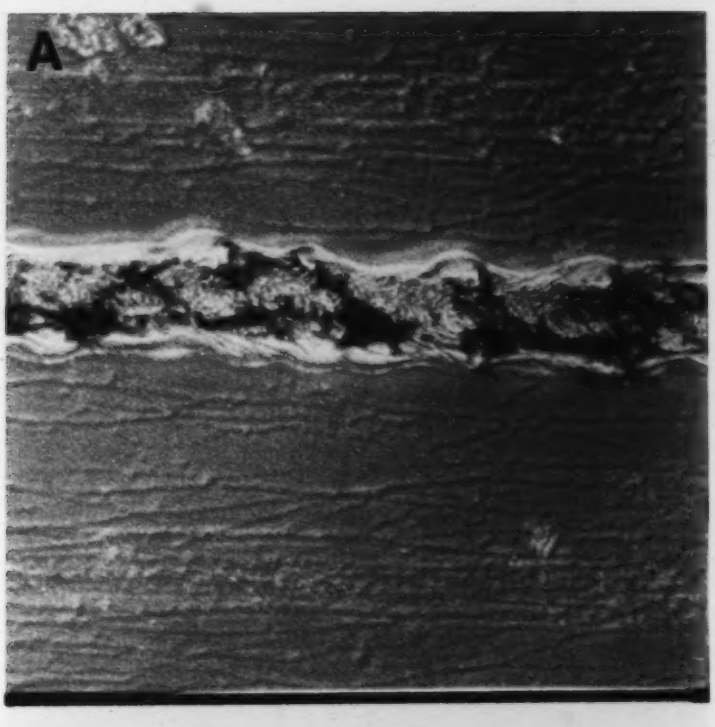
ApoE3(1-183)

ApoE4(1-183)

ApoE(141 - 149)2

Figure 27. Lipid-free ApoE3(1 - 183) Treatment in Centre Cell Body/ proximal Axon-containing Compartment Shows No Apparent Morphological Effects on Distal Axons After 21.0h Treatment

Phase contrast micrograph of rat sympathetic neurons on two tracks before and after 21.0h treatment with lipid-free apoE3(1 - 183) in the centre cell body/proximal axon-containing compartment. Panels are ~ 1.0 mm long. A) Right hand side compartment containing axon cord and branching before treatment, B) Right hand side compartment containing axon cord and branching after 21.0h treatment in centre compartment. Results are representative of three dishes/ treatment and two separate experiments. Magnification = 2,000 X.

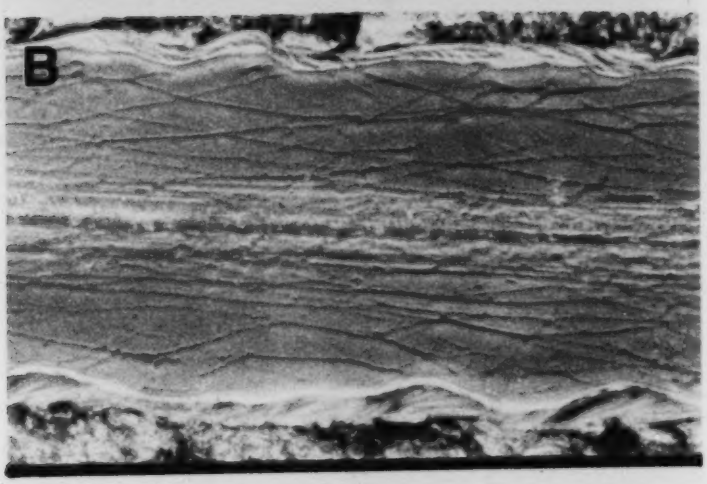

A

B

Figure 28. ApoE4(1 - 183) Treatment in Centre Cell body/proximal Axon-containing Compartment Only Shows No Morphological Changes After 21.0h Treatment But A Decrease in Neurite Branching Can Be Seen After 43.5h Treatment

Phase contrast micrograph of rat sympathetic neurons on a single track before and after treatment with lipid-free apoE4(1 - 183). Panels are ~ 1.0 mm long. A) Right hand side compartment containing distal axons before treatment to centre compartment, B) Representative right hand side compartment containing axon cord and branching 21.0h after treatment to centre compartment, C) Representative right hand side compartment containing axon cord with less branching after 43.5h treatment with lipid-free apoE4(1 - 183) in the centre compartment. Results are representative of three dishes/ treatment and two separate experiments. Magnification = 2,000 X.

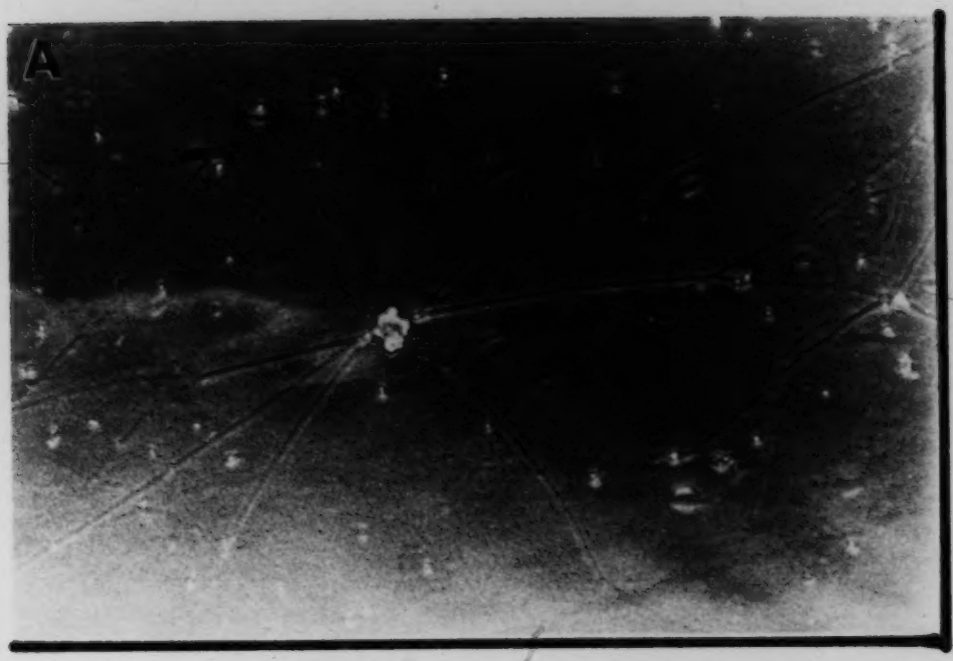
A


B

C

Figure 29. Lipid-free ApoE3(1 - 183) Treatment on Mass Cultures of Rat Sympathetic Neurons Shows No Apparent Morphological Changes After 24h Treatment

Phase contrast micrograph of mass cultures of rat sympathetic neurons before and after treatment with 30 μM lipid-free apoE3(1 - 183). A) Representative neurons of mass cultures before treatment, B) Identical neurons of mass cultures after 24h treatment shows no morphological changes. Results shown representative of 2 separate experiments. Magnification = 2,000 X.


A

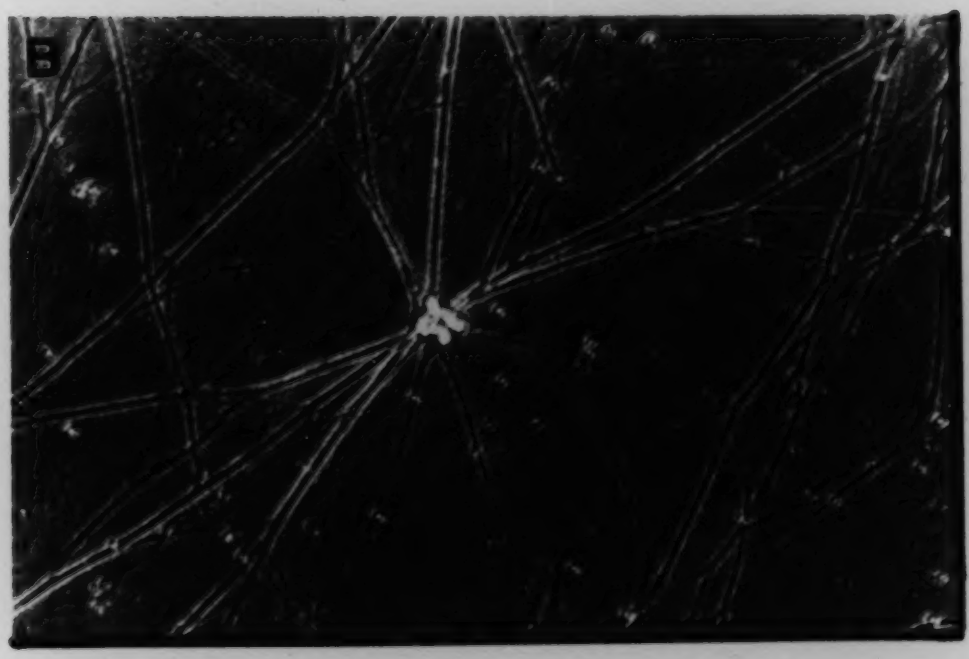
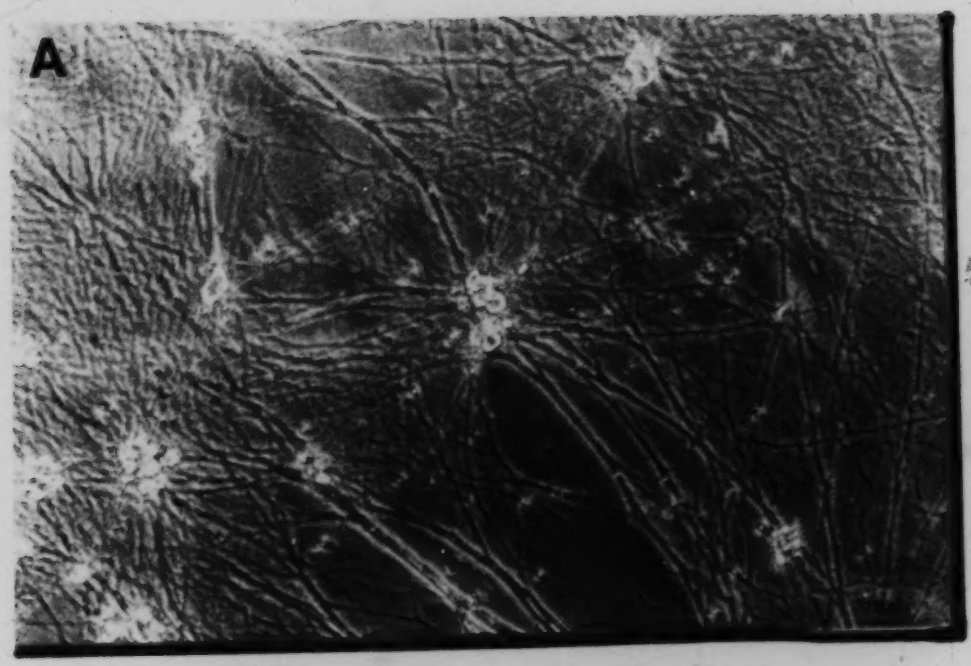

B

Figure 30. Lipid-free ApoE4(1 - 183) Treatment on Mass Cultures of Rat Sympathetic Neurons Shows No Morphological Changes After 24h Treatment

Phase contrast micrograph of mass culture of rat sympathetic neurons before and after treatment with 30 μM lipid-free apoE4(1 - 183). A) Mass culture before treatment, B) Mass culture after 24h treatment. Results shown representative of 2 separate experiments. Magnification = 2,000 X.


A

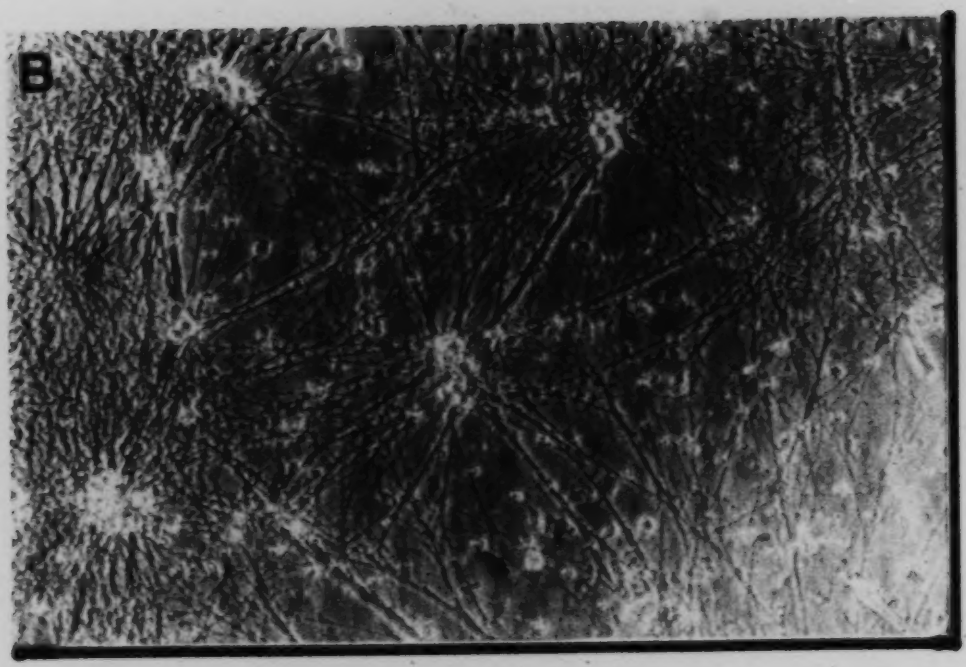
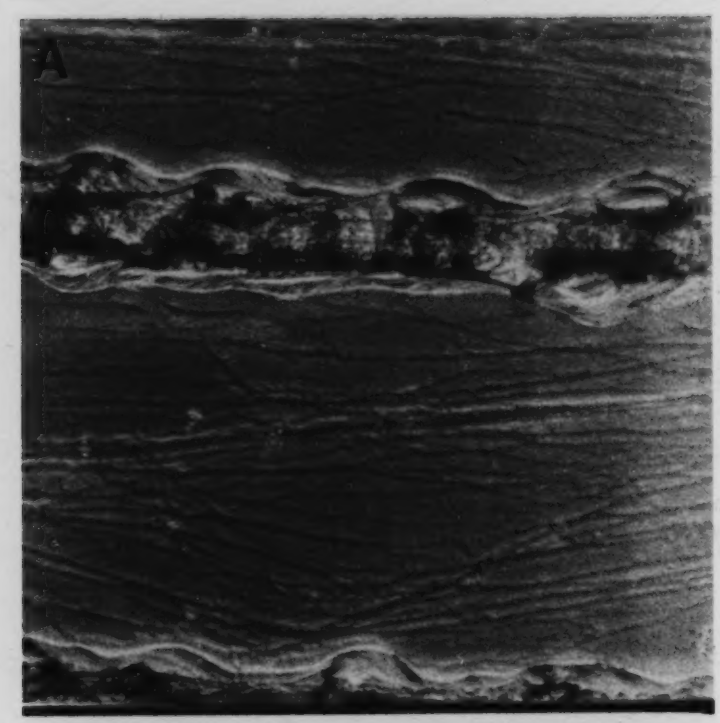

B

Figure 31. Bovine Serum Albumin (BSA) Treatment on Side Compartments Shows No Morphological Changes on Distal Axons After 21h Treatment

Phase contrast micrograph of rat sympathetic neurons on two tracks before and after treatment with 16 μM BSA on distal axon-containing compartments of the compartmented culture. Panels are ~ 1.0 mm long. A) Representative right hand side compartment with distal axons before treatment, B) Representative right hand side compartment after 21.0h treatment. Results shown representative of 3 separate experiments. Magnification = 2,000 X.


A

B

# IV.1 DISCUSSION

## IV.1.1 SUMMARY

Primary cultures of rat sympathetic neurons were used to study the effects of a synthetic tandem repeat peptide, apoE(141 - 149)$^2$, which incorporates a portion of the receptor binding domain of apoE. This synthetic peptide was applied to either the distal axon-containing compartments or the cell body/proximal axon-containing compartment of the compartmented culture to determine if the actions of apoE(141 - 149)$^2$ were specifically limited to the axons alone, to the cell bodies, or to both. It was found that the distal axons were completely destroyed after 24 h treatment in the side compartments only. Surprisingly when the apoE(141 - 149)$^2$ peptide was left on these same axons for 48 h, new neurite sprouting was observed around the degenerated axons. When this synthetic peptide was applied to the centre compartment, both cell bodies/ proximal axons and distal axons in the side compartments remained intact and no cell death was observed. Mass cultures of primary rat sympathetic neurons appeared to be protected in some way against the destructive effects of apoE(141 - 149)$^2$ . These results will be discussed in detail below.

## IV.1.2 RECOMBINANT N-TERMINAL APOE(1 - 183)

Recombinant N-terminal apoE(1 - 183) proteins were also used in these peptide experiments as it had been previously shown that this truncated form of apoE retained almost all the same properties of full-length apoE (Vogel *et al.* 1985, Fisher *et al.* 1997). Three such properties are: 1) ability to bind to a lipid source, such as DMPC, and form apoE(1 - 183)/DMPC discs (Fisher *et al.* 1997), 2) these discs were able to compete with $^{125}$I-LDL particles for LDL binding sites on human skin fibroblasts (Fisher *et al.* 1997) and, 3) when N-terminal apoE(1 - 183) was in a lipid-free form it retained it's three dimensional structure and did not aggregate (Fisher *et al.* 1997). Although this truncated form of apoE does not possess the C-terminus, which contains the lipid binding domain, Raussens and coworkers (1998) demonstrated that the N-terminus forms a strong association to DMPC.

It is possible that the rat sympathetic neurons, or their receptors, may recognize the truncated form of apoE as a foreign particle and inhibit any interaction between protein and receptor. This potential problem must be considered since lipid-free N-terminal apoE3(1 - 183) and apoE4(1 - 183), as well as a lipid-free synthetic tandem repeat peptide of one small section of the apoE protein, were used. Further studies using full-length apoE3 and apoE4 alone, or in association with HDL, or an HDL-like particle, should be undertaken to validate the use of a truncated form of apoE in association with an artificial lipid particle such as DMPC discs.

### IV.1.3 PRIMARY CULTURES OF RAT SYMPATHETIC NEURONS AND APOE(141 - 149)$^2$

It has been shown previously that specific synthetic apoE peptides can exert either cytostatic or cytotoxic effects on T-cells *in vitro* (Clay *et al.* 1995, Tolar *et al.* 1997). The apoE(141 - 149)$^2$ tandem repeat peptide is one of the synthetic peptides that was able to cause a decrease in cell viability when applied to chicken sympathetic neurons, chicken cortical neurons, and rat hippocampal neurons (Tolar *et al.* 1997). Two main questions arise regarding the decrease in viability seen in these three different neuronal cultures: 1) do the apoE(141 - 149)$^2$ peptides exert their effects on both axons and cells bodies or just on the axons alone or the cell bodies only, and 2) are the effects seen with apoE(141 - 149)$^2$ specific to these neurons *in vitro*?

To address the first question the compartmented culture was used. As stated earlier, the compartmented culture is an ideal system for studying the effects of apoE(141 - 149)$^2$ in either the side distal axon-containing compartment or the centre cell body/proximal axon-containing compartment. When apoE(141 - 149)$^2$ peptides were added to the side compartments of 2-week-old cultures a significant decrease in mitochondrial function was seen between these cultures and the control group indicating that these peptides are causing a decrease in mitochondrial activity when added to the distal axon-containing compartment. These results agree with previous studies describing decreased viability in chicken sympathetic neurons and rat hippocampal neurons treated with apoE(141 - 149)$^2$ (Tolar *et al.* 1997).

Surprisingly, when this synthetic tandem repeat peptide was applied to all three compartments or to the side compartments only, no significant difference in mitochondrial function of the cell bodies/proximal axons in the centre compartment was observed between the control group and the apoE(141 - 149)$^2$ treated group. Previous studies done with mass cultures of chicken sympathetic neurons or rat hippocampal neurons showed decreased cell viability when both cell bodies and axons were exposed to apoE(141 - 149)$^2$ (Crutcher *et al.* 1994, Tolar *et al.* 1997). When cell bodies/proximal axons of rat sympathetic neurons were exposed to the toxic peptides they did not exhibit any decrease in mitochondrial activity. It appears that apoE(141 - 149)$^2$ may be acting on the distal axons only or some component of the distal axons. It could also be that mature cell bodies/proximal axons are protected from the actions of the apoE(141 - 149)$^2$ peptides in an as yet unidentified manner.

One possible explanation for protection of neurons focuses on the idea that the toxic peptides are interacting with a receptor or protein present in the distal axons but may be non-existent in the cell bodies. One such protective element may be LRP. In support of this idea, pretreatment of chicken sympathetic neurons with either RAP, a endoplasmic reticulum protein that associates with LRP, or anti-LRP antibody prevented neurotoxic events from occurring when apoE(141 - 149)$^2$ peptides were added (Tolar *et al.* 1997). This suggests that LRP may be involved in the interactions between the toxic peptides and cultures of chicken sympathetic neurons and rat hippocampal neurons. It is not known if or where LRP resides in rat sympathetic neurons.

Therefore, it remains plausible that LRP may be absent from the cell bodies and consequently the cell bodies and thus, the neurons themselves, are protected against the cytotoxic activities of apoE(141 - 149)$^2$.

Another possible explanation may be that the cell bodies are secreting a protective molecule into the centre compartment that inhibits the degenerative effects of the apoE(141 - 149)$^2$ peptides. Although this possibility seems unlikely, since neuronal cell bodies have not been reported to secrete molecules, it cannot be ruled out at this time. This remains to be examined.

Compartmented cultures treated with anti-NGF antibody showed a decrease in mitochondrial activity in both the sides and the centre compartment. A decrease in mitochondrial activity was expected, as rat sympathetic neurons are dependent on NGF for survival (Levi-Montalcini and Booker, 1960).

### IV.1.4 MASS CULTURES OF RAT SYMPATHETIC NEURONS AND APOE(141 - 149)$^2$

Although axonal beading and subsequently cell death are a consequence of apoE(141 - 149)$^2$ treatment in mass cultures of chicken sympathetic neurons, no significant difference (P= 0.8) was observed in mass cultures of rat sympathetic neurons when treated with the same concentration of apoE(141 - 149)$^2$. Gross appearance of mass cultures 24 h after treatment with apoE(141 - 149)$^2$ showed no apparent changes in morphology.

Previous studies, done with cultures of chicken sympathetic neurons, were notably different from the rat sympathetic neuronal cultures used here (Tolar *et al.* 1997). Whereas the chicken sympathetic neuronal cultures were 20 h old when treated with apoE(141 - 149)$^2$, the rat sympathetic neurons were two weeks old before treatment and the difference in culture material was significant. The axons of chicken sympathetic neurons appeared sparse (Tolar *et al.* 1997) while the 2-week-old rat sympathetic neurons had extremely dense axons. This difference in culture material may explain why 8 μM apoE(141 - 149)$^2$ was not enough to cause a difference in mitochondrial function in mass cultures of rat sympathetic neurons and yet a significant decrease in cell viability was seen when this same concentration was applied to the chicken sympathetic neurons (Tolar *et al.* 1997). When the mass cultures of rat sympathetic neurons were treated with 16 μM apoE(141 - 149)$^2$ a decrease in mitochondrial function was seen after 24 and 48 h. It should be noted that the control group also exhibited a decrease in mitochondrial function after 48 h treatment, but this could be the result of exposure to 5 ng/ml NGF for the 48 h. Primary cultures of rat sympathetic neurons require a higher concentration of NGF for optimal growth. Most importantly, the trend seen with the apoE(141 - 149)$^2$ remained the same for both the 24 and 48 h treatment period. The trend of decreased mitochondrial activity seen with 16 μM apoE(141 - 149)$^2$ over the 48 h time period indicated that the mass cultures did not recover from this higher concentrated application of toxic peptides.

## IV.1.5 APOE3(1 - 183) AND MASS CULTURES OF RAT SYMPATHETIC NEURONS

Many studies have been done over the past few years looking at the differential effects of apoE3 and apoE4 on neuronal cultures (Nathan *et al.* 1994, Nathan *et al.* 1995, DeMattos *et al.* 1998). It has been reported that apoE3 enhances, while apoE4 inhibits, neurite outgrowth (Nathan *et al.* 1994). When lipid-free apoE3(1 - 183) was added to mass cultures of rat sympathetic neurons no significant increase (P= 0.6) in mitochondrial activity was observed. There are considerable differences between the primary cultures of rat sympathetic neurons used here and previous studies with either rabbit dorsal root ganglion (DRG) neurons, (Nathan *et al.* 1994), or a murine neuroblastoma cell line (Neuro-2a) (Nathan *et al.* 1995). In the case of the DRG neurons, application of β-VLDL has been shown to stimulate neurite branching (Nathan *et al.* 1994). With the addition of rabbit apoE, which is similar to human apoE3, to β-VLDL an increase in neurite extension was observed concurrent with a decrease in neurite branching (Nathan *et al.* 1994). These cultures had been deprived of serum for 20 h prior to the addition of apoE-enriched β-VLDL. This deprivation results in the upregulation of LDLr or LRP (Nathan *et al.* 1994). In contrast, rat serum was supplied to the mass cultures of rat sympathetic neurons up to the time of treatment. In this case, receptor upregulation prior to treatments was most likely not stimulated and therefore may explain why apoE3(1 - 183) treatment showed no apparent effect on mitochondrial activity.

Human full-length apoE3/β-VLDL was then added to rabbit DRG resulting in the same effects seen with rabbit apoE/β-VLDL: increased outgrowth of neurites and decreased branching. However, when human full-length apoE4/β-VLDL was supplied to rabbit DRG a reduction of both neurite outgrowth and branching was observed (Nathan *et al.* 1994). This suggests that human apoE4 may have an inhibitory effect on neurite outgrowth and branching of DRG neurons. Similar results were obtained with Neuro-2a cells (Nathan *et al.* 1995). In both cases, full-length apoE3 or apoE4 was supplied to the cultures either associated with a lipid source or in addition to the β-VLDL. In contrast, the rat sympathetic neurons received lipid-free apoE3(1-183).

As stated earlier, it is believe that apoE must be associated with a lipid particle for cell-mediated endocytosis to occur. Experiments to address this were attempted using the apoE(1 - 183)/DMPC discs but due to technical problems were unable to be interpreted . Further study is needed to address this. Lipid-free apoE3(1 - 183) may not have had an effect on the mass cultures of rat sympathetic neurons because the apoE was not associated with lipid and therefore could not bind to the receptor and ultimately be taken up by the cells.

## V.1 APOE(141 - 149)$^2$ AND APOE(61 - 69)$^2$ TREATMENTS ON AXOTOMIZED CULTURES OF RAT SYMPATHETIC NEURONS

As expected, NGF and apoE(61 - 69)$^2$ treated groups had no effect on neurite extension over a 48 h time period, but apoE(141 - 149)$^2$ tandem repeat

peptide completely destroyed the axons in both side compartments within 24 h. Surprisingly, when these cytotoxic peptides were left on the degenerated axons for 48 h, new neurite sprouting could be seen. This result was unexpected since previous studies utilizing chicken sympathetic neurons showed considerable cell death by 24 h in cultures treated with apoE(141 - 149)$^2$ (Crutcher *et al.* 1994, Tolar *et al.* 1997).

The fact that the axons treated with apoE(141 - 149)$^2$ began to show regeneration of new neurites after 48 h in culture should be addressed. It is possible that once the axons began to degrade they released proteases or peptidases, that hydrolyze the peptide bonds between the amino acids of apoE(141 - 149)$^2$ and therefore, destroy the peptide. Once these bonds are broken, the α-helical structure, positive charge spacing, and any potential interaction with the neurons are lost. A second possible explanation could be that the axons began to degenerate and released compound(s) that bind to the peptide inhibiting further degenerative actions. These two possibilities for new neurite regeneration remain to be examined.

The control peptides used, apoE(61 - 69)$^2$, which do not possess the evenly spaced positive charges on their α-helical structure necessary for interaction with the receptor, are not affected by putative proteases or peptidases because they do not induce degeneration of the axons.

To ensure that the rat sympathetic neurons were not protecting themselves through some unknown mechanism, the old media in the dishes treated with apoE(141 - 149)$^2$ was removed and the dishes were given fresh

cytotoxic peptide. Neurite regeneration was measured the following day. Again complete neurite degeneration could be seen. This data suggests that a continuous supply of apoE peptides may be necessary in order to see an eventual decrease in cell viability in older cultures. One should keep in mind that the effects seen *in vitro* may not necessarily be true when studying whole animals *in vivo*. The actions of these toxic peptides on a PNS cell may not be applicable when examining a CNS cell, although, Tolar and coworkers (1997) used primary cultures of rat hippocampal neurons from the CNS and saw the same results as chicken sympathetic neurons. This suggests that apoE(141 - 149)$^2$ may have the same actions on CNS cells as they do on PNS cells.

A continuous supply of toxic peptide may have an important role physiologically as studies done with AD subjects show an increase in apoE production with concurrent increases in apoE accumulation in the CNS (Roses, 1995, Hahne *et al.* 1997). Since AD appears to involve inflammation, and is a progressive disease, the production of apoE protein in the CSF may also increase over time. This increase may lead to more apoE protein available to bind to Aβ peptide, resulting in more fragments of apoE being produced (Castao *et al.* 1995). Finally, with a continuous supply of apoE fragments, the possibility of toxic interaction with axons in the brain increases.

What is most intriguing, is that the cell bodies/proximal axons of rat sympathetic neurons treated with apoE(141 - 149)$^2$ in either the sides only or centre compartment only showed healthy, stable cell bodies and intact proximal neurites in the centre compartment 24 h after treatment. It is possible

that there was no effect seen in the centre compartment because the peptide was only able to interact with something in the distal axons and not in the centre compartment, as stated earlier. Even more puzzling, is the fact that the proximal axons in the centre compartment remained intact even when apoE(141 - 149)$^2$ was added. If distal axons are destroyed in the side compartments when treated with the toxic peptides, then a natural assumption would be that the proximal axons in the centre compartment would also be destroyed. Since the proximal axons were not destroyed, this poses a question of whether the proximal axons are protected in some way because of their proximity to the cell bodies in this compartment, or do the distal axons contain something that is lacking in the cell bodies/proximal axons that is interacting with the peptides? Further studies need to be done to answer these questions.

### V.1. 1 ANTI-NGF ANTIBODY TREATMENT ON AXOTOMIZED CULTURES OF RAT SYMPATHETIC NEURONS

Rat sympathetic neurons are dependent on NGF for survival (Levi-Montalcini and Booker, 1960). When the axotomized cultures were supplied with anti-NGF antibody on day 4 post axotomy, a decrease in neurite regeneration could be seen over the next two days.

## V.1. 2 APOE4(1 - 183) TREATMENT ON AXOTOMIZED NEURONAL CULTURES OF RAT SYMPATHETIC NEURONS

As mentioned earlier, it is believed that apoE3 has positive, enhancing effects on neurite outgrowth whereas apoE4 has negative, inhibitory effects on neurites *in vitro* (Nathan *et al.* 1994, 1995). When apoE3(1 - 183) was added to mass cultures no significant effect could be seen. Lipid-free apoE4(1 - 183) was added to day two post axotomy cultures and measurements taken for two days. The neurites appeared to grow even better than the dishes treated with either NGF or apoE(61 - 69)$^2$. One dish per treatment group for both the control and apoE(61 - 69)$^2$ groups was removed from the sample size after one day post-treatment since the neurites had extended into the furthest end of the side compartment and could no longer be measured for neurite regeneration. By 48 h it appeared that the neurites treated with apoE4(1 - 183) were slowing their outgrowth. This may not be surprising since phase micrographs of 43.5 h cultures show a more granulated appearance and as a result this could interfere with neurite extension. These are unexpected results since previous studies done using apoE4 showed a decrease in neurite outgrowth (Nathan *et al.* 1994, 1995) and no mention of increasing granulation.

## V.1. 3 TREATMENTS ON CELL BODIES/PROXIMAL AXONS OF AXOTOMIZED CULTURES OF RAT SYMPATHETIC NEURONS

The following five treatments were supplied to the cell body/proximal axon-containing compartment: 1) base media only, 2) apoE(61 - 69)$^2$, 3) apoE(141 - 149)$^2$, 4) apoE3(1 - 183), and 5) apoE4(1 - 183) and neurite extension measured in the side compartments for three days. As seen in Figure 26, Chapter III, neurite extension was not affected in any way by these treatments in the centre compartment. This experiment supports the notion that the cell bodies may be protective in some way to the destructive effects of the apoE(141 - 149)$^2$ peptides seen in the distal axon-containing compartment. What is unusual is that by 43.5 h exposure to apoE4(1 - 183) a decrease in neurite branching is evident and yet by 43.5 h exposure to the cytotoxic peptide, no difference could be seen in neurite branching. When apoE3(1 - 183) was supplied to the side compartments or to all three compartments, a decrease in neurite extension could be seen. These same cultures displayed no difference in neurite branching when compared to those treated with apoE(61 - 69)$^2$ (Results not shown). Could it be that the longer, 22 kDa apoE4(1 - 183) protein has an effect on neurite branching as proposed by Nathan and coworkers (1994) and that rather than stimulating neurite outgrowth and inhibiting branching, apoE3(1 - 183) decreased outgrowth and had no visible effect on branching?

Taken together these results suggest that apoE(141 - 149)$^2$ and the 22 kDa apoE4(1 - 183) fragment may have devastating results on distal axons only

while cell bodies are protected against these effects. The question of what sort of protection is occurring in primary cultures of rat sympathetic neurons needs to be studied further.

# VI CONCLUSIONS

Alzheimer's disease is not a single entity with respect to either causation or symptoms, but rather a collection of diseases each emerging from different genetic causes (Roses, 1995, Crutcher *et al.* 1997). Various labs throughout the world have generated convincing data that present different theories as to the causation of AD. One such theory states that the N-terminal fragment of apoE, found in both control and AD patients, may have the ability to induce neurotoxicity on CNS neurons of AD patients (Crutcher, et al, 1997). This theory is based on previous studies which have shown that both synthetic peptides and fragments of apoE exhibit cytotoxic effects *in vitro* (Clay, et al, 1995, Crutcher, et al, 1991).

A synthetic tandem repeat peptide of a small portion of apoE, apoE(141 - 149)$^2$, was utilized in this work to determine possible cytotoxic effects of fragments of apoE on primary cultures of rat sympathetic neurons. A compartmented culture system was used to test the effects of apoE(141 - 149)$^2$ either on the distal axons only or on the cell bodies/proximal axons of rat sympathetic neurons. It was found that apoE(141 - 149)$^2$ had degenerative effects on distal axons only.

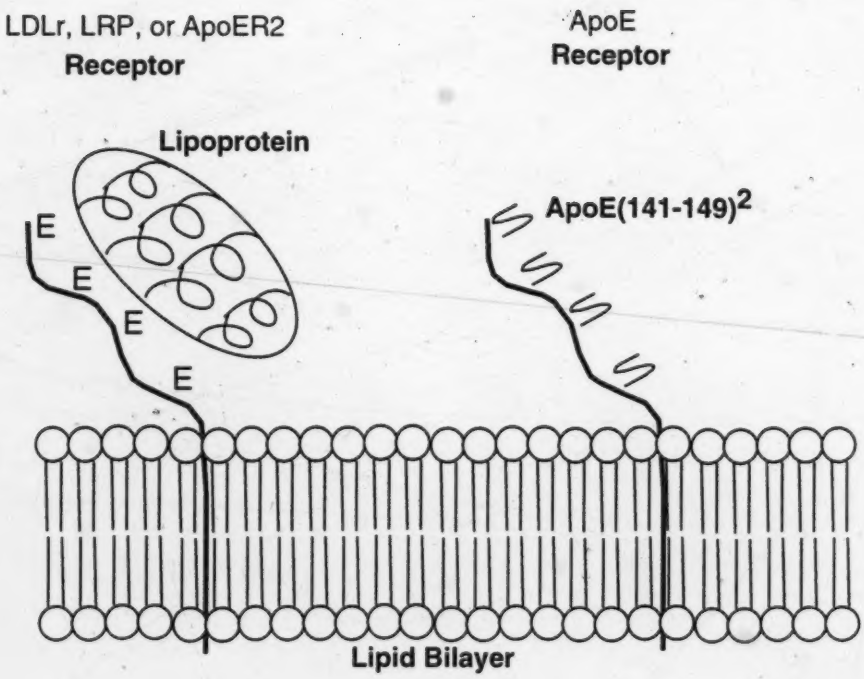
Although it has been shown that apoE must be associated with a lipid particle for receptor-mediated endocytosis to occur (Weisgraber, 1994), it is conceivable that apoE peptides or the N-terminal fragment of apoE is capable of interacting with one of the members of the LDLr family (Tolar, *et al*, 1997 ).

Dyer and coworkers (1991) demonstrated that more than one apoE(141 - 155)$^2$ tandem repeat peptide was capable of binding to both the LDLr and LRP through ionic interactions. Multiplicity of apoE protein and apoE(141 - 155)$^2$ was also reported to be an essential feature of receptor interactions (Clay *et al*, 1995). It is possible then that multiple copies of apoE(141 - 149)$^2$ synthetic tandem repeat peptide could also bind to or interact with apoE receptors. These peptides exhibit at least two appropriately spaced clusters of positively charged residues on an α-helical structure and binding of more than one peptide mimics the binding of more than one apoE protein/receptor. Figure 32 shows the possible interaction between either the apoE(141 - 149)$^2$ or the N-terminal fragment of apoE and an apoE receptor. Two questions that should be addressed are: 1) Which receptor, of the LDLr family, could be interacting with the apoE(141 - 149)$^2$ peptide or the N-terminal apoE4 that leads to neurite degeneration? and, 2) Why is there an apparent lack of effect on cell bodies and proximal neurites, in the centre compartment of the compartmented culture, when treated with apoE(141 - 149)$^2$? These two questions are critical to understanding the proposed interactions between the N-terminal fragment of apoE and AD.

Although it is inappropriate to assume that if one thing occurs in the PNS then naturally this same outcome is expected in the CNS. It may be possible though to infer that by using a PNS model system *in vitro* one can envision similar results occurring in the CNS *in vivo,* since apoE protein and apoE fragments are found in both systems. Although most of the studies done with

Figure 32. Proposed Model of Interaction Between ApoE(141 - 149)$^2$ and ApoE Receptors

Four apoE proteins, in association with a lipoprotein particle, bind to an apoE receptor such as the LDLr, LRP, or ApoER2 (Left receptor), then undergo cell mediated endocytosis. Four apoE(141 - 149)$^2$ synthetic tandem repeat peptides (S shape) may bind to the apoE receptor (Right receptor), in a similar manner stimulating a putative intracellular signal leading to degradation of the distal axons.


LDLr, LRP, or ApoER2
Receptor
ApoE
Receptor
Lipoprotein
E
E
E
E
ApoE(141-149)2
Lipid Bilayer

apoE fragments have focused on neuronal cells of the PNS, there are at least three reasons why apoE fragments are believed to cause devastating effects on neurons of the CNS. Firstly, apoE fragments have been localized in both CSF and hippocampal sections of the brain (Jordan *et al.* 1998), secondly, C-terminal fragments of apoE have been found bound to amyloids A and L and amyloid plaques in the brain (Castao *et al.* 1995), and thirdly, peptide studies done with rat hippocampal neurons of the CNS *in vitro,* have led to the same results as studies done with chicken sympathetic neurons of the PNS (Crutcher *et al.* 1994, Tolar *et al.* 1997).

It was not the intent of this work to simply focus on apoE peptide activities and rat sympathetic neurons. The purpose was to expand on limited reasoning and venture into the possibilities of physiologically relevant apoE peptides and how they may be affecting neurons of the CNS. This naturally leads to the question of what kind of damage apoE peptides or fragments may cause in subjects suffering from AD? Further understanding of apoE fragments, and their effects on CNS neurons and brain function, is absolutely necessary. Once a pathological effect can be determined, damage could possibly be eliminated or at the very least, delayed.

REFERENCES

Aggerbeck, L.P., Wetterau, J.R., Weisgraber, K.H., Shuen-Shang, C. W., and Lindgren F. T: Human Apolipoprotein E3 in Aqueous Solution. *J Biol Chem.* Vol 263, (13), pp. 6249 - 6258, (1988)

Aizawa, Y., Fukatsu, R., Takamaru, Y., Tsuzuki, K., Chiba, K., Kobayashi, N., Fugii, N., Takahata, N: Amino-terminus truncated apolipoprotein E is the major species in amyloid deposits in Alzheimer's disease-affected brains: a possible role for apolipoprotein E in Alzheimer's Disease. *Brain Research*, Vol. 768, pp 208 - 214 (1997)

Avila, E.M., Holdsworth, G., Sasaki, N., Jackson, R.L., and Harmony, J.A.K: Apoprotein E Suppresses Phytohemagglutinin-activated Phospholipid Turnover in Peripheral Blood Mononuclear Cells. *J. Biol. Chem.* Vol. 257. (10), pp. 5900 - 5909 (1982)

Benzing, W.C., and Mufson, E.J: Apolipoprotein E Immunoreactivity Within Neurofibrillary Tangles: Relationship to Tau and Paired Helical Filaments in Alzheimer's Disease. *Exper. Neurology*. Vol. 132, pp 162 - 171 (1995)

Braddock, D.T., Mercurius, K. O., Subramanian, R.M., Dominguez, S. R., Davies, P. F., and Meredith, S.C: Conformationally Specific Enhancement of Receptor-Mediated LDL Binding and Internalization by Peptide Models of a Conserved Anionic N-Terminal Domain of Human ApoE. *Biochem*, 35, pp. 13975 - 84, (1996)

Boyles, J.K., Pitas, R.E., Wilson, E., Mahley, R.W., and Taylor, J.M: Apolipoprotein E Associated with Astrocytic Glia of the Central Nervous System and with Nonmyelinating Glia of the Peripheral Nervous System. *J. Clinical Investigation*, Vol. 76, pp. 1501 - 1513, (1985)

Brown, M.D., Banker, G.A., Hussaini, I.Sm. Gonias, S.L., and Vandenberg, S.R: Low Density lipoprotein receptor-related protein is expressed early and becomes restricted to a somatodendritic domain during neuronal differentiation in culture. *Brain Research,* Vol. 747. pp. 313 - 17, (1997)

Brown, M.S., and Goldstein, J.L: Receptor-Mediated Control of Cholesterol Metabolism. *Science*, Vol. 191. pp 150 - 154, (1976)

Brown, M.S. and Goldstein, J.L: A Receptor-Mediated Pathway for Cholesterol Homeostasis. *Science*, Vol. 232. pp 34 - 47, (1986)

Boyles, J.K., Zoeliner, C.D., Anderson, L.J., Kosik, L.M., Pitas, R.E., Weisgraber, K.H., Hui, D.Y., Mahley, R.W., Geibicke-Haerter, P.J., Ingatius, M.J., and Shooter, E.M: A Role for Apolipoprotein E, Apolipoprotein A-1, and Low Density Lipoprotein Receptors in Cholesterol Transport During Regeneration and Remyelination of the Rat Sciatic Nerve. *J. Clin. Invest.* Vol. 83, pp 1015 - 1031. (!989)

Campenot, R.B: Compartmented Culture Analysis of Nerve Growth. "Cell-Cell Interactions, A Practical Approach", Edited by B.R. Stevenson and D.L. Paul., Oxford University Press. pp 275 - 298 (1992)

Campenot, R.B: Regeneration of Neurites in Long-Term Cultures of Sympathetic Neurons Deprived of Nerve Growth Factor. *Science*, Vol. 214, pp 579 - 581. (1981)

Campenot, R.B., Walji, A.H., and Draker, D.D: Effects of Sphingosine, Staurosporine, and Phorbol Ester on Neurites of Rat Sympathetic Neurons Growing in Compartmented Cultures. *J. of Neurosci.* Vol 11. (4) pp 1126 - 1139, (1991)

Cardin, A.D., Hobuyoshi Hirose, Blankenship, D.T., Jackson, R.L., and Harmony J.A.K: Binding of a High Reactive Heparin to Human Apolipoprotein E: Identification of Two Heparin-Binding Domains. *Biochemical and Biophysical Research Communications*. Vol. 134, No. 2. Pp 783 - 789 (1986)

Carlsson, J., Armstrong, V.W., Reiber, H., Felgenhauer, K., and Seidel, D: Clinical Relevance of the Quantification of Apolipoprotein E in Cerebrospinal Fluid. *Clinica Chimica Acta*. Vol. 196, pp 167 - 176 (1991)

Castao, E.M., Prelli, F., Pras, M., and Frangione, B: Apolipoprotein E Carboxy-fragments are Complexed to Amyloids A and L. *J. Biol. Chem*. Vol. 270, No. 29. Pp 17610 - 17615 (1995)

Chang, J.Y., Brown, S., Cytosine Arabinoside Differentially Alters Survival and Neurite Outgrowth of Neuronal PC12 Cells. *Biochem and Biophysical Research Comm*. Vol. 218. Pp 753 - 758 (1996)

Clay, M.A., Anantharamaiah, G.M., Mistry, J.J., Balasubramaniam, A., and Harmony, J.A.K: Localization of a Domain in Apolipoprotein E with Both Cytostatic and Cytotoxic Activity. *Biochemistry*, Vol. 34, pp 11142 - 11151 (1995)

Crutcher, K.A: On the Relationship Between Inflammation, Apolipoprotein E and Alzheimer's Disease. Internet Conference on Alzheimer's Disease Poster, http://crutcherlab.med.uc.edu/AD.htm. (1998)

Crutcher, K.A., Tolar, M., Harmony J.A.K., and Marques, M.A.: A New Hypothesis for the Role of Apolipoprotein E in Alzheimer's Disease Pathology. *Alzheimer's Disease: Biology, Diagnosis and Therapeutics.* Edited by Iqbal, K., Winblad, B., Nishimura, T., Takeda, M., and Wisniewski, H.M. Chp. 69. pp 543 - 552 (1997)

Crutcher, K.A., Clay, M.A., Scott, S.A., Tian, X., Tolar, M., and Harmony, J.A.K: Neurite Degeneration Elicited by Apolipoprotein E Peptides. *Experimental Neurology*. Vol. 130, pp 120 - 126 (1994)

DeMattos, R.B., Curtiss, L.K., and Williams, D.L: A Minimally Lipidated Form of Cell-derived Apolipoprotein E Exhibits Isoform-specific Stimulation of Neurite Outgrowth in the Absence of Exogenous Lipids and Lipoproteins. *J Biol. Chem.* Vol 273. (7), pp 4206 - 12 (1998)

Diccianni, M.B., Lilly-Stauderman, M., McLean, L.R., Balasubramaniam, M., and Harmony, J.A.K: Heparin Prevents the Binding of Phospholipase $A_2$ to Phospholipid Micelles: Importance of the Amino-Terminus. *Biochemistry*, Vol 30. pp 9090 - 9097 (1991)

Deckwerth, T.L., and Johnson, E.M., Jr: Temporal Analysis of Events Associated with Programmed Cell Death (Apoptosis) of Sympathetic Neurons Deprived of Nerve Growth Factor. *J. of Cell Biol.* Vol. 123 (5), pp 1207 - 22 (1993),

Dessi, F., Pollard, H., Moreau, J., Ben-Ari, Y., and Charriaut-Marlangue, C: Cytosine Arabinoside Induces Apoptosis in Cerebellar Neurons in Culture. *J. of Neurochem.* (64), 1980 - 87 (1995)

Dong, L., Wilson, C., Wardell, M.R., Simmons, T., Mahley, R.W., Weisgraber, K.H., and Agard, D.A: Human Apolipoprotein E. *J Biol. Chem.* Vol 269 (35) pp 22358 - 65 (1994)

Dyer, C.A., Cistola, D.P., Parry, G.C., and Curtiss, L.K: Structural Features of Synthetic Peptides of Apolipoprotein E That Bind the LDL Receptor. *J. of Lipid Research.* Vol. 36. Pp 80 - 88 (1995)

Dyer, C.A., and Curtiss, L.K: A Synthetic Peptide Mimic of Plasma Apolipoprotein E that Binds the LDL Receptor. *J. Biol. Chem.* Vol. 266, No. 34. pp 22803 - 22806 (1991)

Dyer, C.A., Smith. R.S., and Curtiss, L.K: Only Multimers of a Synthetic Peptide of Human Apolipoprotein E are Biologically Active. *J. Biol. Chem.* Vol. 266, No. 23, pp 15009 - 15015 (1991)

Elshourbagy, N.A., Liao, W.S., Mahley, R.W., and Taylor, J.M: Apolipoprotein E mRNA is Abundant in the Brain and Adrenals, As Well As in the Liver, and is Present in Other Peripheral Tissues of Rats and Marmosets. *Proc. Natl. Acad. Sci. USA* Vol. 82 (1) pp. 203 - 207 (1985)

Fagan, A.M., Bu, G., Sun, Y., Daugherty, A., and Holtzman, D.M: ApoE-containing High Density Lipoprotein Promotes Neurite Outgrowth and is a Ligand for the Low Density Lipoprotein Receptor-Related Protein. *J. Biol. Chem.* Vol. 271, No. 47. pp 30121 - 25 (1996)

Fisher, C.A., Wang, J., Francis, G.A., Sykes, B.D., Kay, C.M. and Ryan, R.O: Bacterial Overexpression, Isotope Enrichment, and NMR Analysis of the N-terminal Domain of Human Apolipoprotein E. *Biochem. Cell Biol.* Vol. 75, pp 45 - 53 (1997)

Guillaume, D., Bertrand, P., Dea, D., Davignon, J., and Poirier, J: ApoE and Low-Density Lipoprotein Binding and Internalization in Primary Cultures of Rat Astrocytes: Isoform-specific Alterations. *J of Neurochem.* Vol. 66, pp. 2410 - 18 (1996)

Hahne, S., Nordstedt, C., Ahlin, A., and Nyback, H: Levels of Cerebrospinal Fluid Apolipoprotein E in Patients with Alzheimer's Disease and Healthy Controls. *Neuroscience Letters*, Vol. 224. Pp 99 - 102 (1997)

Handelmann, G.E., Boyles, J.K., Weisgraber, K.H., Mahley, R.W., and Pitas, R.E: Effects of Apolipoprotein E, β-Very Low Density Lipoproteins, and Cholesterol on the Extension of Neurites by Rabbit Dorsal Root Ganglion Neurons *in Vitro*. *J. of Lipid Research*. Vol. 33. Pp 1677 - 1688 (1977)

Havel, R.J., and Kane, J.P: Primary Dysbetalipoproteinemia: Predominance of a Specific Apoprotein Species in Triglyceride-rich Lipoproteins. *Proc. Natl. Acad. Sci. USA* Vol. 70 (7) pp. 2015 - 2019 (1973)

Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H., and Stanley, K.K., Surface Location and High Affinity for Calcium of a 500-kd Liver Membrane Protein Closely Related to the LDL-receptor Suggest a Physiological Role as Lipoprotein Receptor *EMBO J.* Vol. 7, No. 13, pp 4119 - 4127 (1988)

Herz, J. and Willnow, T.E: Functions of the LDL Receptor Gene Family. *Ann. New York Acad. Sci.* Vol. 737 pp. 14 - 19 (1994)

Higgins, G.A., Large, C.H., Rupniak, H.T., and Barnes, J.C: Apolipoprotein E and Alzheimer's Disease: A Review of Recent Studies. *Pharmacol Biochem Behav.* Vol. 56, No. 4 pp 675 - 685 (1997)

Hussain, M.M., Maxfield, F.R., Mas-Oliva, J., Tabas, I., Ji, ZS., Innerarity, T.L., and Mahley, R.W: Clearance of Chylomicron Remnants by the Low Density Lipoprotein Receptor-related Protein/alpha2-macroglobulin Receptor. *J. Biol. Chem.* Vol. 266 (21) pp. 13936 - 13940 (1991)

Ignatius, M.J., Gebicke-Harter, P.J., Pate Sken, J.H., Schilling, J.W., Weisgraber, K.H., Mahley, R.W., and Shotter, E.M: Expression of Apolipoprotein E During Nerve Degeneration and Regeneration. *Proc. Natl. Acad. Sci. USA*. Vol. 83, pp 1125 - 1129 (1986)

Innerarity, T.L., Friedlander, E.J., Rall, S.C. Jr., Weisgraber, K.H., and Mahley, R.W: The Receptor-binding Domain of Human Apolipoprotein E. *J. Biol. Chem*. Vol. 258, No. 20. Pp 12341 - 12347 (1983)

Innerarity, T.L., and Mahley, R.W: Enhanced Binding by Cultured Human Fibroblasts of Apolipoprotein E-Containing Lipoproteins as Compared with Low Density Lipoproteins. *Biochemistry* Vol. 17, No. 8 pp 1440 - 1447 (1978)

Innerarity, T.L. Pitas, R.E. and Mahley, R.W: Binding of Arginine-rich (E) Apoprotein after Recombination with Phospholipid Vesicles to the Low Density Lipoprotein Receptors of Fibroblasts. *J. Biol. Chem*. Vol. 254, No. 10. pp 4186 - 4190 (1979)

Innerarity T.L., Weisgraber, K.H., Arnold, K.S., Rall, S.C.Jr., and Mahley, R.W: Normalization of Receptor Binding of Apolipoprotein E2. Evidence for Modulation of the Binding Site Conformation. *J. of Biol. Chem.* Vol. 259 (11) pp. 7261 - 7267 (1984)

Jordan, J., Galindo, M.F., Miller, R.J., Reardon, C.A., Getz, G.S., and LaDu, M.J: Isoform-Specific Effect of Apolipoprotein E on Cell Survival and β-Amyloid-Induced Toxicity in Rat Hippocampal Pyramidal neuronal Cultures. *J. of Neuroscience.* Vol. 18, No. 1, pp 195 - 204 (1998)

Kim, D-H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H-J., Suzuki, H., Kondo, H., Saeki, S., and Yamamoto, T: Human Apolipoprotein E Receptor 2. *J. Biol. Chem.* Vol. 271, No. 14, pp 8373 - 8380 (1996)

Lalazar, A., Weisgraber, K.H., Rall, S.C.Jr., Giladi, H., Innerarity, T.L., Levanon, A.Z., Boyles, J.K., Amit, B., Gorecki, M., Mahley, R.W., and Vogel, T: Site-specific Mutagenesis of Human Apolipoprotein E. *J. Biol. Chem.* Vol 263, No. 8, pp 3542 - 45 (1988)

Levi-Montalcini, R., and Booker, B: Excessive Growth of the Sympathetic Ganglia Evoked by a Protein Isolated From Mouse Salivary Glands. *Proc. Natl. Acad. Sci. USA.* Vol. 46. pp. 373 - 391 (1960)

Mahley, R.W., Nathan, B.P., Bellosta, S., and Pitas, R.E: Apolipoprotein E: Impact of Cytoskeletal Stability in Neurons and the Relationship to Alzheimer's Disease. *Current Opinion in Lipidology* Vol. 6. pp. 86 - 91 (1996)

Mahley, R.W., Innerarity, T.L. Pitas, R.E., Weisgraber, K.H. and Gross, E: Inhibition of Lipoprotein Binding to Cell Surface Receptors of Fibroblasts Following Selective Modification of Arginyl Residues in Arginine-rich and B Apoproteins. *J. of Biol. Chem.* Vol. 252 (20) pp. 7279 - 7287 (1977)

Mahley, R.W., Innerarity, T.L., Rall, S.C. Jr., and Weisgraber, K.H: Lipoproteins of Special Significance in Arteriosclerosis. Insights Provided by Studies of Type III Hyperlipoproteinemia. *Ann. New York Acad. Sci.* Vol. 454. pp. 209 - 221 (1985)

Mahley, R.W., and Weisgraber, K.H: Canine Lipoproteins and Atherosclerosis. I. Isolation and Characterization of Plasma Lipoproteins from Control Dogs. *Circulation Research* Vol. 35 (5) pp. 713 - 721 (1974)

Mahley, R.W., Weisgraber, K.H., and Innerarity, T.L: Canine Lipoproteins and Atherosclerosis. II. Characterization of the Plasma Lipoproteins Associated with Atherogenic and Nonatherogenic Hyperlipidemia. *Circulation Research* Vol. 35 (5) pp. 722 - 733 (1974)

Marques, M.A., Tolar, M., Harmony, J.A.K., and Crutcher, K.A: A Thrombin cleavage Fragment of Apolipoprotein E Exhibits Isoform-Specific Neurotoxicity. *Neuroreport* Vol. 7, pp 2529 - 2532 (1996)

Mims, M.P., Darnule, A.T., Tovar, R.W., Pownall, H.J., Sparrow, D.A., Sparrow, J.T., Via, D.P., and Smith, L.C: A Nonexchangeable Apolipoprotein E Peptide That Mediates Binding to the Low Density Lipoprotein Receptor. *J Biol. Chem.* Vol 269, No. 32, pp 20539 - 47 (1994)

Muller, H.W. Ignatius, M.J., Hangen, D.H., and Shooter, E.M: Expression of Specific Sheath Cell Proteins During Peripheral Nerve Growth and Regeneration in Mammals. *J. of Cell Biol.* Vol. 102. pp 393 - 402 (1986)

Nathan, B.P., Bellosta, S., Sanan, D.A., Weisgraber, K.H., Mahley, R.W., and Pitas, R.E: Differential Effects of Apolipoprotein E3 and E4 on Neuronal Growth *In Vitro. Science* Vol. 264. pp 850 - 852 (1994)

Nathan, B.P., Chang, K-C., Bellosta, S., Brisch, E., G.E., N., Mahley, R.W., and Pitas, R.E: The Inhibitory Effect of Apolipoprotein E4 on Neurite Outgrowth is Associated with Microtubule Depolymerization. *J. Biol. Chem.* Vol 270, No. 34, pp 19791 - 19799 (1995)

Nikoulin, I.R., and Curtiss, L.K: An Apolipoprotein E Synthetic Peptide Targets to Lipoproteins in Plasma and Mediates Both Cellular Lipoprotein Interactions *In Vitro* and Acute Clearance of Cholesterol-rich Lipoproteins *In Vivo*. *J. Clin. Invest.* Vol. 101, No. 1. Pp 223 - 234 (1998)

Pitas, R.E., Innerarity, T.L., and Mahley, R.W: Cell surface receptor binding of phospholipid-protein complexes containing different ratios of receptor-active and -inactive E apoprotein. *J. Biol. Chem.* Vol 255, No. 11, pp 5454 - 5460, (1980)

Poirier, J: Apolipoprotein E in Animal Models of CNS Injury and in Alzheimer's Disease. *Tips in Neuroscience (TINS)*, Vol. 17, No. 12, pp 525 - 530 (1994)

Posse de Chaves, E.I., Rusinol, A.E., Vance, D.E., Campenot, R.B., and Vance, J.E: Role of Lipoproteins in the Delivery of Lipids to Axons During Axonal Regeneration. *J. Biol. Chem.* Vol. 272, No. 40, pp 30766 - 30773 (1997)

Rall, S.C., Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W: Structural Basis for Receptor Binding Heterogeneity of Apolipoprotein E From Type III Hyperlipoproteinemic Subjects. *Proc. Natl. Acad. Sci. USA* Vol. 79, pp. 4696 - 4700 (1982)

Raussens, V., Fisher, C.A., Goormaghtigh, E., Ryan, R.O., and Ruysschaert, J-M: The Low Density Lipoprotein Receptor Active Conformation of Apolipoprotein E. *J of Biol. Chem.* Vol. 273. No. 40 pp. 25825 - 25830 (1998)

Rebeck, G.W., Reiter, J.S., Strickland, D.K., and Hyman, B.T: Apolipoprotein E in Sporadic Alzheimer's Disease Allelic Variation and Receptor Interactions. *Neuron*, Vol. 11, pp 575 - 580 (1993)

Roses, A.D: Perspective: On the Metabolism of Apolipoprotein E and the Alzheimer Diseases. *Experimental Neurology*, Vol. 132, pp 149 - 156 (1995)

Roses, A.D., and Saunders, A.M: Apolipoprotein E, Alzheimer's Disease, and Recovery from Brain Stress. *Annals New York Academy of Sciences.* Vol. 826, pp 200 - 212 (1997)

Roth, R.I., Jackson, R.L., Pownall, J. H., and Gotto, A.M. Jr: Interaction of Plasma 'Arginine-Rich' Apolipoprotein with Dimyristoylphosphatidylcholine. *Biochemistry* Vol. 16, pp 5030 - 5036 (1977)

Sanan, D.A., Weisgraber, K.H., Russell, S.J., Mahley, R.W., Huang, D., Saunders, A., Schmechel, D., Wisniewski, T., Frangione, B., Roses, A.D., and Strittmatter, W.J: Apolipoprotein E Associates with β Amyloid Peptide of Alzheimer's Disease to Form Novel Monofibrils. *J. Clin. Invest.* Vol. 94, pp 860 - 869 (1994)

Shore, V.G., and Shore, B: Heterogeneity of Human Plasma Very Low Density Lipoproteins. Separation of Species Differing in Protein Components. *Biochemistry*. Vol. 12, No. 3, pp. 502 - 507 (1973)

Snipes, G.J., McGuire, C.B., Norden, J.J., and Freeman, J.A: Nerve Injury Stimulates the Secretion of Apolipoprotein E by Nonneuronal Cells. *Proc. Natl. Acad. Sci. USA*. Vol. 83, pp 1130 - 1134 (1986)

Sparrow, J.T., Sparrow, D.A., Culwell, A.R., and Gotto, A.M. Jr: Apolipoprotein E: Phospholipid Binding Studies with Synthetic Peptides Containing the Putative Receptor Binding Region. *Biochemistry* Vol. 24, pp 6984 - 6988 (1985)

Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Migliorini, M., and Argraves, W.S: Sequence Identity Between the α2-macroglobulin Receptor and Low Density Lipoprotein Receptor-related Protein Suggests That This Molecule is a Multifunctional Receptor. *J. Biol. Chem*. Vol. 265, pp. 17401 - 17404 (1990)

Strittmatter, W.J., Saunders, A.M., Schmechel, D., Perick-Vance, M., Enghild, J., Salvesen, G.S., and Roses, A.D: Apolipoprotein E: High-avidity Binding to Beta-amyloid and Increased Frequency of Type 4 Allele in Late-onset Familial Alzheimer Disease. *Proc. Natl. Acad. Sci. USA*. Vol. 90 (5) pp. 1977 - 1981 (1993)

Strittmatter, W. J., and Roses, A.D: Apolipoprotein E and Alzheimer's Disease *Proc. Natl. Acad. Sci. USA* Vol 92, pp 4725 - 4727 (1995)

Strittmatter, W.J., *et al.*, Isoform-Specific Interactions of Apolipoprotein E with Microtubule Associated Protein Tau: Implications for Alzheimer's Disease. *Proc. Natl. Acad. Sci. USA*. Vol. 91, pp 11183 - 11186 (1994)

Tolar, M., Marques, M.A., Harmony, J.A.K., and Crutcher, K.A: Neurotoxicity of the 22 kDa Thrombin-Cleavage Fragment of Apolipoprotein E and Related Synthetic Peptides Is Receptor-Mediated. *J. of Neuroscience*. Vol. 17, No. 15. Pp 5678 - 5686 (1997)

Vogel, T., Weisgraber, K.H.Zeevi, M.I., Ben-Artzi, H., Levanon, A.Z., Rall, S.C. Jr., Innerarity, T.L., Hui, D.Y., Taylor, J.M., Kanner, D., Yavin, Z., Amit, B., Aviv, H., Gorecki, M., and Mahley, R.W: Human Apolipoprotein E Expression in *Escherichia coli*: Structural and Functional Identity of the Bacterially Produced Protein with Plasma Apolipoprotein E. *Proc. Natl. Acad. Sci. USA*. Vol. 82, pp 8696 - 8700 (1985)

Wang, Z-S., Luebbe, P., Gruenstein, E., and Zemlan, F: Apolipoprotein E (ApoE) Peptide Regulates Tau Phosphorylation Via Two Different Signaling Pathways. *J. of Neurosci. Research*. Vol. 51, pp 658 - 665 (1998)

Weisgraber, K.H: Apolipoprotein E Distribution Among Human Plasma Lipoproteins: Role of the Cysteine-Arginine Interchange at Residue 112. *J. Lipid Research*, Vol. 31. pp 1503 - 1511 (1990)

Weisgraber, K.H: Apolipoprotein E: Structure-Function Relationships. *Advances in Protein Chemistry*, Vol. 45. pp 249 - 310 (1994)

Weisgraber, K.H., Innerarity, T.L., Harder, K.J., Mahley, R.W., Milne, R.W., Marcel, Y.L., and Sparrow, J.T: The Receptor-Binding Domain of Human Apolipoprotein E. *J. Biol. Chem.* Vol. 258, No. 20 pp 12348 - 12354 (1983)

Weisgraber, K.H., and Mahley, R.W: Human Apolipoprotein E: The Alzheimer's Disease Connection. *FASEB J.* Vol. 10, pp 1485 - 1494 (1996)

Weisgraber, K.H., and Mahley, R.W: Subfractionation of HDL Lipoproteins by Heparin-Sepharose Affinity Chromatography. *J of Lipid Research* Vol 21, pp 316 - 325, (1980)

Weisgraber, K.H., Rall, S.C., Jr., Mahley, R.W., Milne, R.W., Marcel, Y.L., and Sparrow, J.T: Human Apolipoprotein E. *J. Biol. Chem.* Vol 261 No. 5, pp 2068 - 76 (1986)

Westerlund, J.A., and Weisgraber, K.H: Discrete Carboxyl-Terminal Segments of Apolipoprotein E Mediate Lipoprotein Association and Protein Oligomerization. *J. Biol, Chem.* Vol 268, No. 21, pp 15745 - 50 (1993)

Wetterau, J.R., Aggerbeck, L.P., Rall, S.C. Jr., and Weisgraber, K.H: Human ApoE3 in Aqueous Solution. *J. Biol. Chem.* Vol 263, No. 13, pp 6240 - 48 (1988)

Wilson, C., Wardell, M.R., Weisgraber, K.H., Mahley, R.W., and Agard, D.A: Three-Dimensional Structure of the LDL Receptor-Binding Domain of Human Apolipoprotein E. *Science*, Vol. 252. pp 1817 - 1822 (1991)

Wolf, B.B., *et al.*, Characterization and Immunochemical Localization of Low Density Lipoprotein Receptor-Related Protein in Human Brain. *Am. J. Pathol.* Vol. 141, pp 37 - 42 (1992)

Yokoyama, S., Kawai, Y., Tajima, S., and Yamamoto, A. Behavior of Human Apolipoprotein E in Aqueous Solutions and at Interfaces *J. Biol. Chem.* Vol. 260. No. 30, pp 16375 - 16382 (1985)

Yokoyama, S: Self-associated Tetramer of Human Apolipoprotein E Does Not Lead to Its Accumulation on a Lipid Particle. *Biochim. Biophy. Acta.* Vol. 1047 (1) pp. 99 - 101 (1990)

Zannis, V.I., Karathanasis, S.K., Forbes, G.M., and Breslow, J.L. Intra- and extracellular Modifications of Apolipoproteins *Methods in Enzymology* Vol. 128. Pp 690 - 712 (1986)

Zhong-Sheng, J., Dichek, H.L., Miranda, R.D., and Mahley, R.W: Heparan Sulfate Proteoglycans Participate in Hepatic Lipase and Apolipoprotein E-mediated Binding and Uptake of Plasma Lipoproteins, Including High Density Lipoproteins. *J. Biol. Chem.* Vol 272, No. 50 pp 31285 - 31292 (1997)

Zhong-Sheng, J., Pitas, R.E., and Mahley, R.W: Differential Cellular Accumulation/Retention of Apolipoprotein E Mediated by Cell Surface Heparan Sulfate Proteoglycans. *J. Biol. Chem.* Vol. 273, No. 22, pp 13452 - 13460 (1998)